REMARKS

Status of the Claims

Claims 13-15, 18-20, 33-34, and 37 are pending in the present application. Claims 1-12, 16-17, 21-32, and 35-36 are canceled. Claim 13 and 34 are amended. Claim 37 is new. Support for the amendment to claim 13 is found throughout the application as originally filed including on pages 8-9, and 11. Support for new claim 37 is found in claim 13. Support for "retinal ganglion cells" in claims 13 and 24 is found, for example, on page 8, lines 11-15 in combination with page 10, lines 6-9 in the originally filed application. Claim 34 is amended for consistency with claim 13. No new matter is entered by way of this amendment. Reconsideration is respectfully requested.

Statement of the Substance of the Interview

Applicants and Applicants' representatives thank the Examiner for extending the courtesy of an interview on July 1, 2011. The substance of the interview is substantially as described in the Examiner interview summary, which issued on July 8, 2011.

During the interview, the Examiner indicated that claims describing an NGF dosage of 200 to 500 μg/ml are likely sufficient to overcome JP 10-218787 to Okamoto in view of, for example, the unexpected results of the dosage, which was described during the interview. However, the Examiner stated that the pending claims may be rejected under 35 U.S.C. § 103(a) as allegedly obvious in view of PCT Publication No. WO 98/48002 to Lambiase ("Lambiase") in combination with EP 0312208 to Finkenaur *et al.* ("Finkenaur"). These references were previously cited, for example, in the April 29, 2009, Office Action.

In particular, the Examiner indicated that subject matter describing treating posterior tissues of the eye, such as the optic nerve, are allowable. However, the Examiner asserted that the sclera and retina encompass regions, which are not posterior tissues of the eye. Accordingly, the Examiner does not believe that the pending claims are novel and non-obvious in view of Lambiase and Finkenaur.

In an effort to expedite prosecution, Applicants' representative suggested that the claims be amended to specify that an effective amount of topically applied nerve growth factor is increased in the retinal ganglion cells and the optic nerve. The Examiner agreed that this amendment was likely to be non-obvious over Lambiase and Finkenaur.

The claims are amended as described during the interview. For the reasons set forth below, Applicants believe the amended claims are allowable.

Obviousness-Type Double Patenting Issues

Claims 13, 15, 18-20, and 33 remain provisionally rejected on the ground of nonstatutory obviousness-type double patenting as allegedly unpatentable over claims 1, 8-11, and 13 of co-pending U.S. Application No. 12/064,172, *see* Office Action, page 2.

Applicants submit that if a provisional non-statutory obviousness-type double patenting (ODP) rejection is the only rejection remaining in the earlier filed of the two pending applications, while the later-filed application is rejectable on other grounds, the Examiner should withdraw that rejection and permit the earlier-filed application to issue as a patent without a terminal disclaimer, *see* MPEP § 804. The relevant "filing date" for double patenting purposes is the earliest U.S. filing date for patent term calculation.

The instant application has a PCT filing date of January 21, 2000. U.S. Application No. 12/064,172 has a PCT filing date of August 11, 2006. Accordingly, Applicants submit that, in view of the arguments and claim amendments submitted herewith, the only rejection remaining in the instant application is the obviousness type double patenting rejection. Therefore, the Examiner should withdraw the rejection and permit the instant application to proceed to allowance.

Issues under 35 U.S.C. § 103(a)

Okamoto I and Okamoto II

Claims 13-15, 18-20, 33, and 34 remain rejected under 35 U.S.C. § 103(a) as allegedly obvious over JP 10-218787 to Okamoto ("Okamoto I") for the reasons of record, *see* Office Action, pages 3-4. Applicants respectfully traverse.

According to the Examiner, Okamoto teaches methods of treating pathologies of the eye, including glaucoma, by topical administration of Nerve Growth Factor (NGF), *see* page 4 of the Office Action, which issued on July 16, 2010. The Examiner indicates that Okamoto does not describe the specific dosage range as described in the present claims, *see* the instant Office Action, page 3. Nevertheless, the Examiner asserts that an ordinary artisan would have reasonably known that dosages are results-effective variables, which can be optimized.

For the reasons set forth below, Applicants submit that the amended claims are not rendered obvious by Okamoto I. Further, Applicants note that PCT Publication No. WO 98/10785, (Okamoto II), which published on March 19, 1998, includes the subject matter described in Okamoto I plus additional subject matter regarding ophthalmic compositions of neurotrophic factors. Accordingly, the following response will reference the subject matter in Okamoto I and Okamoto II. Applicants note that U.S. Patent No. 6,261,545 corresponds to Okamoto II.

Okamoto I and Okamoto II do not teach or suggest all of the elements of the instant claims

Applicants submit that neither Okamoto I nor Okamoto II teach or suggest all of the elements of the amended claims. In particular, neither Okamoto I nor Okamoto II teach or suggest "topically applying a composition comprising from 200 to 500 μg/ml of nerve growth factor over an ocular surface of the subject to cause an increase in the amount of nerve growth factor in the retinal ganglion cells, and optic nerve, wherein an effective amount of the nerve growth factor is provided to said tissues." There is absolutely no disclosure in Okamoto I or Okamoto II, for example, which expressly states or suggests that NGF reaches the retinal ganglion cells.

The alleged presence of NGF in the optic nerve described in Okamoto I and II does not suggest the presence of an effective amount of NGF in the retinal ganglion cells

Okamoto I and Okamoto II indicate that an NGF drug solution reaches the optic nerve *via* the cornea and an anterior aqueous humor or is scattered in corpus vitreum, *see* paragraph [0052] of the English language machine translation of Okamoto I and column 6, lines 50-54 of U.S. Patent No. 6,261,545. According to Okamoto I and II, the drug solution also reaches the chorioid-suprachoidal space through an iris and a ciliary body, and allegedly reaches the optic nerve where NGF shows an effect, *see* paragraph [0052] of Okamoto I and column 6, lines 59-63 of U.S. Patent No. 6,261,545. Nevertheless, neither Okamoto I nor Okamoto II suggest that an effective amount of NGF reaches all of the posterior target tissues including posterior portions of the retina, such as the retinal ganglion cells.

At the time of the invention, it was uncertain if a topically applied drug, including a large molecule such as NGF, would reach all of the posterior tissues of the eye, including, for example, the posterior portions of the retina. Applicants further submit that at the time of the invention, an

ordinary artisan was aware that even if a topically applied drug could, hypothetically, reach the optic nerve in an effective amount, such an observation would not have suggested that the drug was also present, for example, in the retinal ganglion cells. It was also uncertain how, or if, an effective amount of NGF could reach, for example, the retinal ganglion cells. For instance, it was unclear if NGF could pass by route of the sclera, choroid, choriocapillaris and be capable of permeating the retinal pigment epithelium to reach the posterior portions of the retina. It was also uncertain if NGF passed into the retrobulbar space and the optic nerve head and could be retro-transported by the optic nerve to retinal ganglion cells. In view of the foregoing, Okamoto's disclosures regarding the presence of NGF in the optic nerve would not have reasonably suggested to an ordinary artisan that any dosage of NGF could have reached, for example, the posterior portion of the retina, such as the retinal ganglion cells.

For the Examiner's information, Applicants further note that Okamoto's description that the NGF preparation "is adsorbed from a cornea and transited to an anterior aqueous humor, a part thereof reaches an optic disc", is not possible *see* column 6, lines 51-52 of U.S. Patent No. 6,261,545. Applicants submit that it has been widely demonstrated that NGF does not cross the cornea, *see* Exhibit A, Lambiase *et al.*, *IVOS*, 2005, 46:3800-3806, page 3805, paragraph 2.

Okamoto I and II teach away from the range of NGF specified in the present claims

In *Ex parte Whalen*, 89 USPQ2d 1078, 1083 (BPAI 2008) the limitations in question, molecular weight of a polymer and viscosity of a pharmaceutical composition containing the polymer, were know in the prior art as affecting the properties of the composition. Despite the fact that the properties in question were know to affect the results, **the Board of Appeals held that the invention was not *prima facie* obvious because the prior art suggested that the best results would be achieved outside of the claimed range**. In this regard, the Board of Appeals stated:

> Here, the Examiner has not pointed to any teaching in the cited references, or provided any explanation based on scientific reasoning, that would support the conclusion that those skilled in the art would have considered it obvious to "optimize the prior art compositions by increasing their viscosity to the level recited in the claims. No reason to have done so is apparent to us based on the record. On the contrary, the references all suggest that low viscosity was a

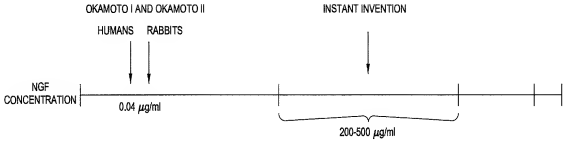
> desired property in embolic compositions. ... Therefore, Evans' preferred composition has a viscosity less than half of that required by the instant claims.

In the present case, Okamoto I teaches that the dosage of neurotrophic factor ranges from 0.1 μg/250 ml (0.0004 μg/ml) to about 100 mg/250 mls (400 mg/ml), *see* paragraph 51 of Okamoto I. Okomoto II teaches that the dosage of neurotrophic factor may range from about 0.0001 to 0.5% w/v (1 to 5000 μg/ml), or 10^{-3} to 2 x 10^{5} μg/l (10^{-6} to 200 μg/ml), particularly preferably 0.0004 to 0.04% w/v (4 to 400 μg/ml) or 10^{-1} to $1x10^{3}$ μg/l (10^{-4} to 1 μg/ml), *see* column 3, lines 39-42 of U.S. Patent No. 6,261,545 .

Applicants note that the above described ranges refer generally to the dosages of any neurotrophic factor including NGF, BNDF (a brain derived neurotrophic factor), CNTF (a ciliary neurotrophic factor), NT-3 (neurotrophin-3), NT-4/5 (neurotrophin-4/5), NT-6, (neurotrophin-6) and derivatives thereof, *see* column 2, lines 54-66 of U.S. Patent No. 6,261,545, column 3, line 40 of U.S. Patent No. 6,261,545, which refers to "the amount of neurotrophic factor", and *see also* paragraph [0051] and paragraph [0012] in Okamoto I.

In contrast to the generalized dosage ranges for neurotrophic factor described in Okamoto I and Okamoto II, the example sections of these documents specifically discuss a dosage for the topical administration of NGF. In particular, the example of Okamoto I and the Test Examples 1 and 2 of Okamoto II, describe topically administering 0.04 μg/ml of NGF to rabbit eyes. Prior to this administration, the intraocular pressure of the rabbit eyes was increased, resulting in an optic nerve disorder. Okamoto I and Okamoto II state that the optic nerve function was recovered in a week. Further, Example 7 of Okamoto II teaches that topical administration of 0.04 μg/ml of NGF to a glaucoma patient resulted in enlargement of a human patient's visual field.

In view of the teachings in Okamoto I and Okamoto II, Applicants submit that a person of ordinary skill in the art at the time of the invention would not have modified the concentration of the NGF preparation from 0.04 μg/ml to 200 to 500 μg/ml since the range of 200 to 500 μg/ml is so much greater than the alleged effective concentration of 0.04 μg/ml described in Okamoto I and Okamoto II, *see* Figure 1 below.



As held in *Ex parte Whalen*, an invention is not *prima facie* obvious if the prior art suggests that the best results would be achieved outside of the claimed range. Here, Okamoto I and Okamoto II allegedly exemplify that lower concentrations of NGF are effective to restore optic nerve damage and to improve the visual field of a glaucoma patient. Accordingly, Applicants submit that an ordinary artisan would not have considered it obvious to optimize the prior art compositions by increasing the NGF concentration to the much higher level recited in the instant claims. In fact, an ordinary artisan would have been discouraged from increasing the dosage and possibly increasing side effects or toxicity.

Unexpected Effects

1. 200 μg/ml of topically administered NGF enhances survival of retinal ganglion cells

Further, the range of NGF described in the present claims results in effects that could not have been expected by an ordinary artisan at the time of the invention. For example, the concentration range of NGF recited in the claims is surprisingly efficacious in enhancing survival of retinal ganglion cells. As indicated in Exhibit B, dosages of 200 μg/ml of NGF effectively protect retinal ganglion cell (RGC) degeneration, which may occur in a glaucomatous retina, *see* Exhibit B, Lambiase *et al.*, *Proc. Natl. Acad. Sci USA*, 2009, 106:13469-13474, *in particular*, pages 13469-13470, bridging paragraph, which states that a significantly higher biologic effect of 200 μg/ml NGF eye drops in protecting RGC loss in rat retinal sections was observed in comparison to 100 μg/ml NGF. *See also* Exhibit C, Colafrancesco *et al.*, *J. Glaucoma*, 2011, 20:100-108, which further demonstrates that ocular administration of 200 μg/ml NGF may be used to protect degenerating retinal ganglion cells.

2. 200-500 μg/ml of topically administered NGF reach the retina and optic nerve

The range of 200-500 μg/ml as described in the pending claims result in further effects, which could not have been expected by an ordinary artisan at the time of the invention. As noted above, the claimed methods require that nerve growth factor, when topically administered, reaches the optic nerve and retina in an effective amount to treat the described diseases. An ordinary artisan, upon reviewing Okamoto I or Okamoto II would not have recognized that this advantageous effect was possible.

Applicants note that the concentration of topically administered NGF exemplified in the Okamoto references, 0.04 μg/ml in human and rabbit, will not reach the optic nerve and retina. According to the available studies on the ocular pharmacokinetics of NGF eye drops, no increase of NGF amounts can be detected in both retina and optic nerve when NGF is administered at 1 μg/ml concentrations (25 times more than the concentration used in Okamoto's examples), *see also* Exhibit D, which describes the passage of NGF through rabbit ocular tissues. Additionally, an accumulation of NGF is not possible because it has been widely demonstrated that NGF is physiologically metabolized. Even at the maximum concentration ever tested (500 μg/ml eye drop), no detectable increase in NGF can be observed after 24 hours.

In contrast to the concentrations of NGF exemplified in Okamoto I and Okamoto II, Exhibit D evidences that an NGF concentration of 200 μg/ml significantly increases the amount of NGF in the retina and the optic nerve over baseline. Concentrations of 1 μg/ml do not increase the amount of NGF in either the retina or the optic nerve over baseline, *see* Exhibit D.

3. 200-500 μg/ml of topically administered NGF crosses the blood ocular barrier

Applicants further submit that NGF crosses the blood ocular barrier when topically administered at a dosage ranging from 200-500 μg/ml. This finding is unexpected. When NGF is administered systemically, NGF does not cross the blood brain barrier. Moreover, NGF does not cross the blood ocular barrier in significant amounts when topically administered at doses outside of the 200-500 μg/ml range. For example, when 10 μg/ml of NGF was topically administered to rats, NGF was not present in the serum, *see* Exhibit A, Lambiase *et al.*, *IVOS*, 2005, 46:3800-3806, in particular Figure 3 and page 3805, right column, paragraph 2. In contrast, when NGF is topically administered at dosages of 200 μg/ml and 500 μg/ml to one eye, NGF is increased in the serum as well as the retina, optic nerve, and sclera of the contralateral

untreated eye of rats, which strongly indicates that NGF passes through the blood-ocular barrier, *see* Exhibit A, pages 3804-3805, bridging paragraph, and page 3805, right column, paragraph 2.

Applicants note that the ability to cross the blood ocular barrier may further contribute to effective amounts of NGF in the retina, in addition to direct passage of NGF through the conjunctiva and sclera and passage through the retro-bulbar space and its retro-transport by the optic nerve to RGS, *see* Exhibit E, Lambiase *et al.*, *Drug News & Perspectives*, 2010, 23:361-367, *in particular* page 365. The ability to cross the blood ocular barrier at the dosages described in the present claims could not have been expected by an ordinary artisan at the time of the invention.

4. Higher concentrations of topically administered NGF are required for therapeutic efficacy

Applicants further submit that an ordinary artisan could not have reasonably expected from Okamoto I and Okamoto II that higher concentrations of topically administered NGF, such as the 200-500 μg/ml concentrations described in the claims, are required to achieve therapeutic efficacy of a variety of ocular diseases including glaucoma. Unexpectedly, topically administered NGF dosages of greater than 100 μg/ml are required to achieve therapeutic efficacy in, for example, optic glioma, glaucoma, and age-related macular degeneration, *see* Exhibit F, Falsini *et al.*, *Neurorehabil Neural Repair*, 2011, 35:512-520, Exhibit G, Chiaretti *et al.*, *Neurorehabil Neural Repair*, 2011, 25:386-390, (electronic publication on February 22, 2011), Exhibit H, Lambiase *et al.*, *Ann Ist Super Sanita*, 2009, 45:439-442 and Exhibit B. *See also* Exhibit E, which is a review article describing the efficacy of topically administered NGF in the treatment of pathologies of the eye and Exhibit I, Sposato *et al.*, *Neuroscience Letters*, 2008, 446:20-24, for a discussion of NGF and NGF-receptors and their role on the optic nerve of rats with experimentally induced elevated intraocular pressure.

An ordinary artisan could not have been reasonably certain that topically administered NGF could have been useful in the treatment of glaucoma from the examples in Okamoto I and Okomoto II.

Animal Model

Applicants submit that a person of ordinary skill in the art at the time of the invention could not have reasonably predicted that topically administered NGF could have been efficaciously used in the treatment of glaucoma from the examples in Okamoto I and Okamoto II. These references use an unpublished animal model, which was obtained by inducing an enormous (60 mmHg) acute increase of intraocular pressure (IOP) by a corneo-scleral sucking device. Applicants submit that such a procedure cannot be considered a model mimicking human glaucoma, which is characterized by a chronic increase of IOP, slightly above the normal levels (21 mmHg), which slowly induces retina and optic nerve progressive damage over several years. Applicants submit that the model of Okamoto resembles an acute ischemic ocular damage instead of typical glaucoma damage. In fact, as a primary parameter of efficacy, Okamato used pupil light reflex reaction, which is never affected in glaucoma, but is a typical sign of optic nerve ischemia or severe trauma. As a secondary parameter, Okamoto used visual evoked potentials (VEPs). Once again, these are constantly affected in severe acute optic nerve injury, but only late and partially affected in glaucoma. Spontaneous recovery of VEPs following ischemic optic nerve damage is frequently observed and shows great variability. Accordingly, in the absence of quantitative data and statistical evaluation, no conclusions on the effectiveness of NGF treatment on the optic nerve injury proposed by Okamoto can be drawn. Moreover, and even more importantly, Okamoto states that "recovery of VEP was… observed", *see* column 8, lines 28-29 of U.S. Patent No. 6,261,545. Applicants submit that recovery of VEP can be observed only in post-ischemic injury, while a spontaneous recovery of VEP is NEVER observed in glaucoma.

In view of the animal model used in Okamoto I and Okamoto II, an ordinary artisan could not have been reasonably certain that any concentration of NGF could have been useful for treating glaucoma.

Human Subject

Okomoto II also describes the effect of NGF in a human glaucoma patient, *see* Example 7 of U.S. Patent No. 6,261,545. However, Applicants submit that an ordinary artisan could not have been reasonably certain from this disclosure that NGF could have been effective in the treatment of glaucoma. Okamoto discloses that he treated only 1 (one) patient with glaucoma with NGF eye drops at a concentration much below that of any published study by the present inventor or others. As noted above, subsequent to Applicant's invention, NGF effects have only been observed at concentrations above 100 μg/ml. Accordingly, Applicants note that Okamoto's claims regarding the efficacy of NGF at 0.04 μg/ml appear unreliable.

Moreover, Okamoto alleges that in order to evaluate the effects of NGF on a single treated patient, he used manual Goldman perimetry. It has been widely proven and published, including in clinical trials, that Goldman perimetry is very much examiner dependent. Goldman perimetry may not be reproducible by another examiner, and it does not have the advantages of a computerized system for storage and comparison to normative data, *see* Exhibit J, Argarwal *et al.*, *Indian J Ophthalmol*, 2000, 48:301-306 and Exhibit K, which includes four scientific abstracts. This is especially true as Okamoto presents data from only 1 (one) patient, therefore, without any kind of statistical analysis.

Additionally, kinetic perimetry may not be as sensitive as static perimetry in detecting early glaucoma defects. In fact, computerized perimetry is unanimously considered the best available technique, providing reliability, sensitivity, reproducibility, and also correlation to retinal ganglion cell loss, *see* Exhibit J.

Other limits and disadvantages of Goldman perimetry include (*see* Exhibits J and K):

1. Direction and speed of stimulus movement are guided by the examiner's hand and, therefore, are difficult to standardize. Thus, the results depend on the examiner's skills and may be confounded by examiner bias (in Okamoto II, it is not disclosed that the exams were performed in a masked fashion as it must be done for any clinical trial, therefore, the examiner bias must be considered of primary importance). Examiner dependence can be associated with poor reproducibility of results.

2. Goldman perimetry results, as being subjectively obtained, are notoriously difficult to quantify, and this is made even more difficult by the lack of standardization of equipment and method.

3. Goldman perimetry does not measure the depth of a scotoma (visual field defect). Because of the pantograph mechanism in the Goldmann instrument, the spatial resolution decreases with increasing eccentricity, which can give rise to a poor cartographic accuracy. Patients who repeat Goldman perimetry over time show an improvement due to a so-called "learning effect." This can be overcome only with computerized examinations when results are confirmed at least three times.

4. There are also other shortcomings of the Goldmann perimeter, such as lack of autocalibration, lack of permanent documentation of the test procedure used to determine individual visual field borders, and the inability to examine the area of 2° around the fixation point.

In view of the foregoing, Applicants submit that ANY conclusion can be drawn by a personal evaluation of a Goldman manual perimetry of a single patient. Accordingly, an ordinary artisan could not have reasonably predicted from Okamoto I or Okamoto II that NGF may be used to efficaciously treat glaucoma.

In view of the foregoing, Applicants submit that the amended claims are not rendered obvious by either Okamoto I or Okamoto II. Accordingly, withdrawal of the rejection is respectfully requested.

Lambiase and Finkenaur

As noted above, the Examiner stated during the interview that the pending claims are rendered obvious by Lambiase in combination with Finkenaur. The Examiner states that the combination of Lambiase and Finkenaur teach topical ocular NGF administration for treating surface and anterior tissues of the eye. The Examiner indicated during the interview that certain regions of the sclera and retina are not posterior eye tissues, but may be considered surface or anterior tissues.

Although Applicants do not agree with the Examiner, the claims are amended in an effort to expedite prosecution. As amended, independent claims 13 and 37 are directed to a method for the treatment of a pathology affecting internal tissues of an eye selected from the group consisting of retinal ganglion cells and optic nerve by topically applying a composition comprising from 200 to 500 μg/ml of nerve growth factor over an ocular surface of the subject to cause an increase in the amount of nerve growth factor in retinal ganglion cells and optic nerve.

The claims further specify the pathologies to be treated, *i.e.*, optic neuritis, glaucoma, maculopathy, retinitis pigmentosa, myopic retinopathy, and macular foramen.

Applicants submit that neither Lambiase nor Finkenaur when considered alone or in combination teach or suggest all of the elements of the amended claims.

Lambiase describes the use of nerve growth factor for the storage of corneas in culture, the *in vitro* production of corneal and conjunctival tissues and the treatment of corneal and conjunctival disease, *see* page 1, lines 5-7 of Lambiase. However, Lambiase fails to describe that nerve growth factor may be topically administered to treat posterior tissues of the eye such as retinal ganglion cells and the optic nerve. Accordingly, Lambiase does not teach or suggest treating pathologies associated with the retinal ganglion cells and/or the optic nerve including optic neuritis, glaucoma, maculopathy, retinitis pigmentosa, myopic retinopathy, and macular foramen as specified in the claims.

Finkenaur does not remedy the deficiencies of Lambiase. Finkenaur merely discloses aqueous gel formulations or viscous solutions for the controlled delivery of growth factors to a wound site in the anterior chamber of the eye, *see* page 2, lines 36-40 of Finkenaur. Further, NGF is only described in the background section of Finkenaur, and is not listed as one of the growth factors used in Finkenaur's methods, *see* line 14 in the background section and claim 4 of Finkenaur. Accordingly, Finkenaur also fails to teach or suggest the treatment of pathologies using topically administered NGF, which pathologies are associated with retinal ganglion cells and/or the optic nerve as described in the instant pending claims.

In view of the foregoing, independent claims 13 and 37 are not rendered obvious by the combination of Lambiase and Finkenaur. Dependent claims 14-15 and 18-20, which incorporate all of the elements of independent claim 13, are also not rendered obvious by these references. Accordingly, Applicants submit that the claims are allowable in view of the prior art.

If the Examiner believes there are any outstanding matters that need to be resolved in the present application, the Examiner is respectfully requested to contact Linda T. Parker, Ph.D., Registration No. 46,046, at 703-205-8052.

CONCLUSION

In view of the above amendment and remarks, Applicant believes the pending application is in condition for allowance.

Should there be any outstanding matters that need to be resolved in the present application, the Examiner is respectfully requested to contact Linda T. Parker, Ph.D., Registration No. 46,046, at the telephone number of the undersigned below to conduct an interview in an effort to expedite prosecution in connection with the present application.

If necessary, the Director is hereby authorized in this, concurrent, and future replies to charge any fees required during the pendency of the above-identified application or credit any overpayment to Deposit Account No. 02-2448.

Dated: JUL 11 2011

Respectfully submitted,

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Attachments

Pharmacokinetics of Conjunctivally Applied Nerve Growth Factor in the Retina and Optic Nerve of Adult Rats

Alessandro Lambiase,[1,2] *Paola Tirassa,*[3] *Alessandra Micera,*[2] *Luigi Aloe,*[3] *and Stefano Bonini*[1,2]

Purpose. Nerve growth factor (NGF) has been shown to inhibit retinal ganglion cell (RGC) degeneration when injected intraocularly in animal models of ocular hypertension, optic nerve transaction, and ischemia. The present study sought to establish the bioavailability of topical NGF to the retina and optic nerve in rats.

Methods. Autoradiography was performed to evaluate whether exogenous ^{125}I-labeled NGF reaches the retina and optic nerve when applied topically to the rat conjunctiva. To quantify NGF levels, a highly specific immunoenzymatic test (ELISA) was performed on the retina, optic nerve, lens, sclera and serum of rats at different time points after administration of NGF (1–500 μg/mL). The physiological activity of topically applied NGF was evaluated by determining retinal brain-derived neurotrophic factor (BDNF) protein and mRNA levels by ELISA and RT-PCR, respectively.

Results. After topical conjunctival administration of NGF, high levels were detected in ocular tissues, including the retina and optic nerve, showing a peak increase 6 hours after administration at a concentration of 200 μg/mL. NGF treatment was associated with an increase in BDNF protein and mRNA levels in rat retina.

Conclusions. These data demonstrate the bioavailability of NGF to the retina and optic nerve in rats when administered topically. These findings justify investigating the clinical effects of topical NGF therapy for treatment of posterior segment diseases. (*Invest Ophthalmol Vis Sci.* 2005;46:3800–3806) DOI:10.1167/iovs.05-0301

Nerve growth factor (NGF) is the best-characterized neurotrophin, known to play a key role in the survival and differentiation of select neurons in the peripheral and central nervous system.[1] Since its discovery in the 1950s, NGF has shown promise in the treatment of progressive neurodegenerative disorders.[1,2] In animals, NGF is known to promote nerve terminal outgrowth and neuron recovery after ischemic, traumatic, and toxic injuries.[1–3] In humans, intracerebral infusions of NGF improved the symptomatology associated with Parkinson's and Alzheimer's disease.[4,5] Particularly in patients with Alzheimer's disease, NGF treatment resulted in cognitive improvement as well as increased cerebral blood flow and electroencephalogram (EEG) changes.[5] Unfortunately, the difficulty of these invasive procedures and the complexity of NGF administration have hindered further progress of controlled clinical studies.[6]

NGF has been shown to act on cells belonging to the visual system.[7] NGF receptors are expressed in the retina of chick embryos, as well as in the retinal pigment epithelium, Müller cells, photoreceptors, and retinal ganglion cells (RGCs) of developing and adult rodents.[8–10] Rat RGCs have been shown not only to express its receptors, but also to transport NGF in a retrograde and anterograde fashion along their axons, which together comprise the optic nerve.[8] In animal models of ocular disease, intraocular administration of NGF improves RGC degeneration after optic nerve transection, ocular ischemia, or induced ocular hypertension.[9–11]

Recently, topical NGF treatment was shown to be a safe and efficient therapy for patients with corneal ulcers or trigeminal nerve impairment.[12] These encouraging findings prompted the present study investigating the potential use of NGF eye drops for the treatment of retinal and/or optic nerve diseases. Time course and dose–response studies were performed to identify the bioavailability of NGF in the retina and optic nerve of rats after topical conjunctival NGF treatment.

Materials and Methods

Animals

Pathogen-free, adult Sprague-Dawley rats (male, 200–250 g) were maintained on a 12-hour light–dark cycle and provided with food and water ad libitum. Regarding housing, care, and experimental procedures, this study conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and to the National Research Council's Ethical Commission on Animal Experimentation (1992) in conformity with national and international laws (EEC council directive 86/609, OJ L 358, 1, December 12, 1987). Moreover, all efforts were made to minimize the animals' suffering and to reduce the number of animals used. All animals were killed while under anesthesia ($n = 117$), their eyes were removed, and ocular tissues (sclera, retina, lens, and optic nerve) and serum were collected and used for biochemical and molecular analyses.

Study Design

Animals were divided into three experimental groups: (1) 25 rats were qualitatively evaluated by autoradiography for the passage of topical NGF eye drops from the ocular surface to the retina and optic nerve; (2) 72 animals were used to (a) quantify physiologic NGF levels in ocular tissues and serum of untreated animals, (b) evaluate whether topical saline administration to the conjunctiva changes NGF levels in ocular tissues and serum of topical saline-treated animals and (c)

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Disclosure: **A. Lambiase,** None; **P. Tirassa,** None; **A. Micera,** None; **L. Aloe,** None; **S. Bonini,** None

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quantify NGF levels in the sclera, retina, lens, optic nerve, and serum of topical NGF-treated animals (10–500 μg/mL) at various time points after dosage; and (3) 20 animals were used to verify whether topical NGF treatment alters NGF and/or BDNF mRNA expression in the retina.

NGF Isolation and Treatment

NGF was isolated from mouse submandibular gland and prepared according to the method of Bocchini and Angeletti.[13] Briefly, the submaxillary glands of adult male mice were explanted under sterile conditions, and the tissues were homogenized, centrifuged, and dialyzed. This aqueous gland extract was then passed through subsequent cellulose columns, thereby separating NGF by adsorption. The first step was gel filtration (Sephadex G-100 column; Roche Diagnostics, Mannheim, Germany) at pH 7.5, in which most of the active NGF was eluted in the 80,000 to 90,000 molecular weight range (designated the G-100 pool). The G-100 pool was then dialyzed at pH 5.0 and fractionated by CM52 cellulose chromatography at pH 5.0. The samples obtained were analyzed by spectrophotometry at a wavelength of 280 nm to identify NGF-containing fractions. Specificity of fractions was determined by Western blot analysis. NGF purity (>95%) was estimated by high-performance liquid chromatography (HPLC; A-progel TSK3000PW-dp 10 mm, 7.5 mm inner diameter 630 cm; TSK, North Bend, WA) column equipped with a guard column calibrated with 40 mg of purified and bioactive murine 2.5S NGF standard. The NGF obtained was then dialyzed and lyophilized under sterile conditions and stored at −20°C until used. Biological activity of purified NGF was evaluated by in vitro stimulation of neurite outgrowth in rat pheochromocytoma PC12 cells over a period of 7 to 14 days.

Subsequently, NGF was dissolved in 0.9% sterile saline in concentrations from 1 to 500 μg/mL. In a masked fashion, rats were treated in one randomly selected eye with one 10-μL dose by topical instillation into the conjunctival fornix. The contralateral eye was used as an internal control.

Autoradiography

NGF was radio-iodinated with ^{125}I-Na (IMS30, 1 mCi; GE Healthcare, Piscataway, NJ) by the chloramine-T procedure and purified by chromatography (Sephadex G-25 column; GE Healthcare, Amersham, Milan, Italy).[14] Specific activity was 1.0 to 1.5 Ci/mmol.

Twenty rats received a 1-μg/mL saline solution containing topical, conjunctivally instilled ^{125}I-NGF eye drops, and the presence of radiolabeled NGF was determined in intraocular tissues after different time intervals (2, 6, 24, and 48 hours after instillation, $n = 5$ at each time point). To assess specific NGF binding, one group of five rats was treated with 100-fold excess of nonradiolabeled, cold NGF.

Autoradiography was performed on 15-μm cryostat sections cut from paraformaldehyde-fixed eyes. Slides were coated with nuclear tracking emulsion (Ilford K2; Ilford Scientific Product, Basildon, UK), and developed (model D19 developer; Eastman Kodak, Rochester, NY). Sections were counterstained with toluidine blue and evaluated by light microscopy (Axiophot; Carl Zeiss Meditec, Jena, Germany).

Pharmacokinetics and Dose–Response Effects of Topical NGF

One control group of eight untreated rats was used to determine the basal, physiologic expression of NGF mRNA and protein in the retina, optic nerve, lens, and sclera, as well as in the serum by an enzyme-linked immunosorbent assay (ELISA) and semiquantitative RT-PCR ELISA.[14,15] A second control group consisted of one group of eight rats treated topically with the saline diluent in one randomly selected eye and killed 6 hours after treatment. NGF mRNA and protein levels were determined in ocular tissues, as just described.

Three concentrations of NGF—10, 200, and 500 μg/mL—were investigated in three experimental groups of eight rats each. In each rabbit, a 10-μL drop of NGF was instilled into the conjunctival fornix of one randomly selected eye. Animals were killed after 6 hours, and intraocular and serum NGF mRNA and protein levels were determined as described earlier.

To investigate the pharmacokinetics of topical NGF, a 10-μL drop of 200 μg/mL NGF was instilled into one eye of 32 animals. After 2, 6, 24, and 48 hours, eight rats were killed, and the NGF-treated and contralateral, untreated eyes were enucleated. NGF levels were determined in ocular tissues and serum as described earlier.

Effect of Topical NGF on Retinal NGF and BDNF mRNA Expression

To evaluate the possible physiological/pharmacological effects of topical NGF treatment on NGF and BDNF synthesis in the retina, 10, 200 or 500 μg/mL of NGF was topically instilled in five rats per dose (total, 15 rats). The animals were killed after 6 hours and treated with NGF, the contralateral eyes were enucleated, and the total RNA was extracted from the retina. This RNA was then used for NGF and BDNF RT-PCR ELISA as described later. The levels of BDNF protein in the retina were also quantified after the procedures for tissue extraction described in the following sections were performed. A group of five saline-treated rats served as the control.

NGF and BDNF Assay

Retina, optic nerve, lens, and sclera were homogenized in sample buffer (described later), and centrifuged at 8500*g* for 30 minutes, and the supernatant was used for NGF ELISA. Polystyrene 96-well immunoplates (Nunc, Roskilde, Denmark) were coated with affinity-purified polyclonal goat anti-NGF antibodies and diluted in 0.05 M carbonate buffer (pH 9.6). Parallel wells were coated with purified goat IgG (Zymed, South San Francisco, CA) for evaluation of nonspecific signal. After an overnight incubation at room temperature and a 2-hour incubation with a blocking buffer (0.05 M carbonate buffer [pH 9.5] 1% BSA), the plates were washed three times with Tris-HCl (pH 7.4; 50 mM), NaCl 200 mM, 0.5% gelatin, and 0.1% Triton X-100. After they were washed, the samples and NGF standard solution were diluted with sample buffer (0.1% Triton X-100, 100 mM Tris-HCl [pH 7.2], 400 mM NaCl, 4 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride [PMSF], 0.2 mM benzethonium chloride, 2 mM benzamidine, 40 U/mL [~0.8

Table 1. Primers Used for the Molecular Study

Gene	Primer	Sequence	Position	Product (bp)	GenBank Accession No.
GAPDH	Biotinylated forward	5′-CACCACCATGGAGAAGGCC-3′	346-365		
	Reverse	5′-GATGGATGCCTTGGCCAGG-3′	517-536	190	M32599
	Digoxygened probe	5′-ACAATCTTGAGTGAGTTGTCATATTTCTCG-3′	451-480		
NGF	Biotinylated forward	5′-TCCACCCACCCAGTCTTCCA-3′	660-679		
	Reverse	5′-GCCTTCCTGCTGAGCACACA-3′	984-1003	343	V00836
	Digoxygened probe	5′-TCGATGCCCCGGCACCCACTCTCAACAGGA-3′	841-871		
BDNF	Biotinylated forward	5′-AGCTGAGCGTGTGTGACAGT-3′	493-512		
	Reverse	5′-TCCATAGTAAGGGCCCGAAC-3′	726-745	252	X55573
	Digoxygened probe	5′-TAACCCATGGCATTACACTTGCTCACGTAG-3′	626-655		

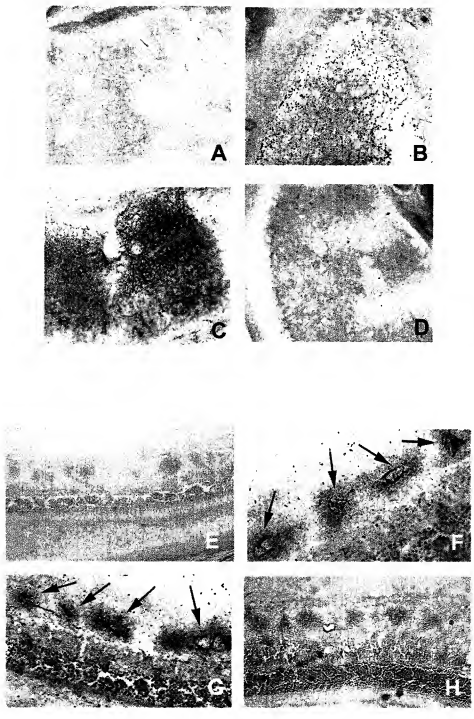

FIGURE 1. ^{125}I-NGF detection in the optic nerve (**B–D**) and in RGCs (**F–H**) by autoradiography counterstained with toluidine blue, 2 (**B**, **F**), 6 (**C**, **G**), and 48 (**D**, **H**) hours after topical conjunctival NGF administration. After 6 hours, the presence of radiolabeled NGF was markedly increased in both the optic nerve (**C**) and in RGCs (**G**, *arrows*). No radiolabeled NGF was detectable 48 hours after application (**D**, **H**). Autoradiography of ocular tissue from rats that received topically a 100-fold excess of nonradiolabeled NGF (cold) is shown in Figure (**A**) and (**E**).

mM] aprotinin, 0.7 mM sodium azide, 0.3 M BSA, and 0.5% gelatin), distributed into the wells and left at room temperature overnight. The plates were then washed three times and incubated with 4 mU/well anti-β-NGF-galactosidase (Roche Diagnostics) for 2 hours at 37°C and, after further washing, 100 μL of substrate solution (4 mg/mL of chlorophenol red [Roche Diagnostics] substrate buffer: 100 mM HEPES, 150 mM NaCl, 2 mM $MgCl_2$, 0.1% sodium azide, and 1% BSA) was added to each well. After an incubation of 2 hours at 37°C, the optical density (OD) was measured at 575 nm with an ELISA reader (Dynatech, Cambridge, MA), and the values of standards and samples were corrected for nonspecific binding. Under these conditions, sensitivity was 3 pg/mL and recovery of NGF ranged from 80% to 90%. Recovery was estimated by adding a known amount of purified NGF to the tissue extracts: the yield of exogenous NGF was calculated by subtracting exogenous from endogenous NGF. Data are presented as picograms per gram wet weight. All assays were performed in triplicate.

BDNF protein levels were determined with an ELISA kit (Promega, Madison, WI), according to the manufacturer's instructions.

RT-PCR ELISA

A standardized RT-PCR ELISA method reported by Tirassa et al.[15,16] was used to evaluate the effects of the treatments on NGF and BDNF mRNA expression levels in the retina.

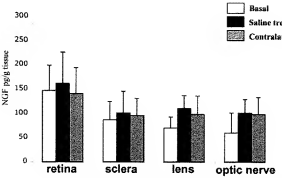

FIGURE 2. Basal physiologic levels of NGF in the ocular tissues of untreated or saline-treated rats. No significant change was observed in either eye group after topical saline treatment.

In this method, multiple sets of primer pairs were used in a coamplification reaction that amplified the target gene of interest within a predetermined range specific for each target. To get semiquantitative results, the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was coamplified with the target gene of interest, to control for variations in product abundance due to differences in individual RT and PCR reaction efficiencies. After amplification, PCR products were detected and measured by ELISA.

Briefly, total RNA was extracted from tissues (TRIzol kit; Invitrogen-Gibco, Grand Island, NY) and complementary DNA (cDNA) was synthesized from 2 μg of RNA with 200 units of M-MLV reverse transcriptase (Promega Italia, Milan, Italy) in 20 μL of total volume reaction containing 250 ng oligo $(dT)_{12\text{-}18}$ primer, 0.5 units RNasin RNase inhibitor, and 0.5 mM dNTP in 5× reaction buffer (250 mM Tris-Cl [pH 8.3], 375 mM KCl, 15 mM $MgCl_2$; 50 mM dithiothreitol [DTT]). The mixture was incubated at 42°C for 1 hour, and the reaction was

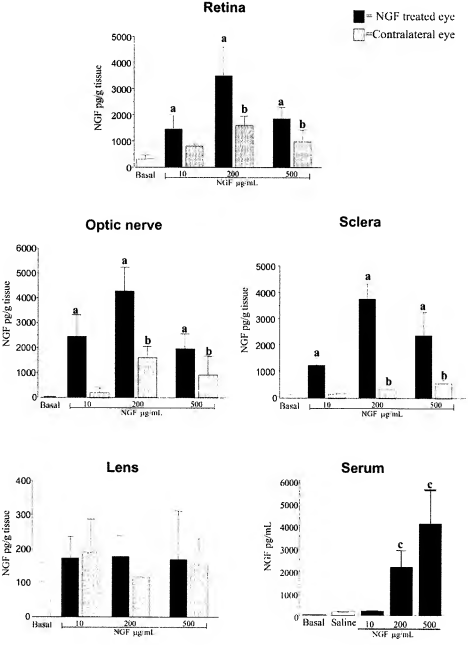

FIGURE 3. The effect of topically administered NGF on intraocular and serum NGF levels. NGF administration resulted in a significant increase of NGF levels in the sclera, retina, and optic nerve, but not the lens, of NGF-treated eyes at all doses tested, with a maximum effect occurring at 200 μg/mL NGF (a: $P < 0.01$ versus parallel control, untreated eyes and versus internally controlled, contralateral eyes). NGF levels in the retina, optic nerve, and sclera of the contralateral eye were also affected at the 200- and 500-μg/mL doses, but not at the 10-μg/mL dose (b: $P < 0.01$ versus parallel control, untreated eyes). NGF also increased in the serum of rats compared with both untreated and saline-treated animals (c: $P < 0.01$).

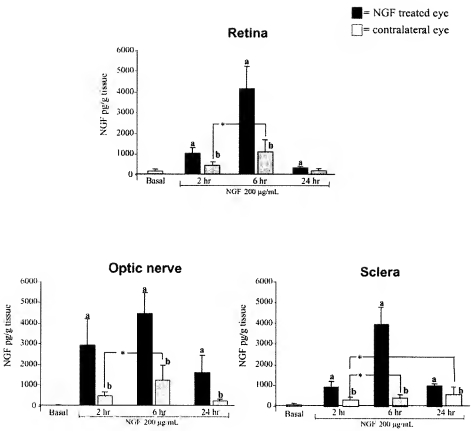

FIGURE 4. Pharmacokinetics after topical conjunctival instillation of a 10-μL drop of NGF (200 μg/mL). NGF levels were significantly higher in the retina, optic nerve, and sclera of NGF-treated eyes (a: $P < 0.01$ versus both parallel control, untreated eyes and versus internal control, contralateral eyes) and contralateral eyes (b: $P < 0.01$ versus parallel control, untreated eyes). NGF levels were high at 2 hours, peaked at 6 hours, and waned at 24 hours. NGF levels in contralateral eye tissues at different time points was significant compared with basal levels (*$P < 0.05$).

terminated with a further incubation at 95°C for 5 minutes. PCR amplification was achieved with 5′ biotinylated primers, to generate biotinylated PCR products detectable by digoxygenin-labeled probes in an immunoenzymatic assay (ELISA). The sequences of primers and probes are listed in Table 1.

cDNA was mixed with 5 μL 10× buffer, 200 μM dNTPs, 1.5 mM $MgCl_2$, 2.5 units of *Taq* DNA polymerase (Promega), and primers in a final volume of 50 μL. A sample containing all reaction reagents except cDNA was used as a PCR negative control in all amplifications. Ten microliters of RT mixture without enzyme was used as an additional PCR negative control. The mixes were incubated for 30 cycles (denaturation, 1 minute at 95°C; annealing, 1 minute at 55°C, extension 2 minutes at 72°C) in a PCR system (GeneAmp 9700; Applied Biosystems, Inc. [ABI], Foster City, CA). Biotinylated PCR products diluted in PBS containing 3% bovine serum albumin (PBSB) were distributed in triplicate (100 μL/well) onto avidin-coated microplates and incubated 1 hour at room temperature. After incubation and denaturation with 0.25 M NaOH, the plates were incubated with 100 μL/well of 4 pmol/mL digoxygenin (DIG)-labeled probes in DIG easy hybridization buffer (Roche Diagnostics) for 2 hours at 42°C. After washes, anti-DIG POD-coupled antibody (Roche Diagnostics) was added (1:1000 in PBSB), and samples were incubated 1 hour at 37°C. The reaction was developed by TMB (3,3′,5,5′-tetramethylbenzidine; 0.6 mg in citrate buffer [pH 5.0]) and blocked after 30 minutes with 2 M HCl. The amount of amplified products was measured as OD at 450/690 nm (OD 450/690) with an ELISA reader (model 5000; Dynatech). GAPDH optic density (OD 450/690) levels were used to normalize for the relative differences in sample size, integrity of the individual RNA and variations in reverse transcription efficiency.

Data Analysis

Statistical analysis was performed on computer (SuperANOVA; Abacus Concepts, Inc., Berkeley, CA) and the Tukey-Kramer post hoc comparison. $P < 0.05$ was considered statistically significant.

RESULTS

Autoradiography demonstrated radiolabeled NGF in the conjunctiva, sclera, choroid, retina, and optic nerve, but not in the corneal stroma. Levels achieved maximum absorption 6 hours after treatment. However, as early as 2 hours, radiolabeled NGF was detected in the optic nerve (Fig. 1B), and to a lesser extent in the retina (Fig. 1F), whereas after 6 hours, radiolabeled NGF was localized in all ocular tissues including the optic nerve (Fig. 1C) and the retina (Fig. 1G). Radiolabeled NGF was not detectable in any tissue 48 hours after treatment (Figs. 1D, 1H). No labeling was observed when a 100-fold excess of cold, nonradiolabeled NGF was administered as a control of autoradiography specificity (Figs. 1A, 1E). However, the possibility that autoradiography reflected levels of NGF metabolites rather than the native and active NGF cannot be excluded.

In untreated animals, NGF protein was expressed by all ocular tissues (basal values), with a higher expression in the retina (147 ± 52 pg/g) than in the optic nerve (60 ± 41 pg/g). No significant changes in NGF were observed in the contralateral untreated eye or after saline treatment (Fig. 2).

After one dose of topical NGF eye drops, a significant increase in NGF protein was detected in the serum and in all ocular tissues, except lens (Fig. 3). These increases were statistically significant compared with contralateral eyes and the parallel control groups (untreated and saline-treated) at all NGF doses administered. In particular, 200 μg/mL NGF resulted in a twofold increase in NGF levels in both the retina and optic nerve 6 hours after treatment ($P < 0.01$; Fig. 3).

A markedly lower but statistically significant increase of NGF content in the retina, optic nerve and sclera was also observed in the contralateral eye of rats receiving the higher doses, 200 and 500 μg/mL, compared with tissue levels in untreated animals ($P < 0.01$, Fig. 3). This finding probably

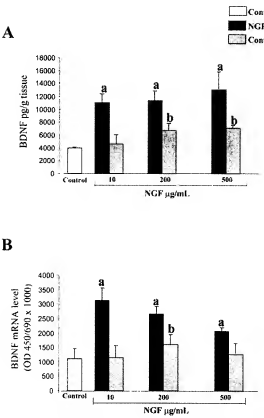

FIGURE 5. Effects of topical conjunctival instillation of a 10-μL drop of NGF (200 μg/mL) on the expression of BDNF protein (A) and mRNA (B) in the retina of NGF-treated and contralateral eyes at all doses used (a: $P < 0.01$ versus both parallel control, untreated eyes and versus internal control, contralateral eyes; b: $P < 0.05$ versus parallel control, untreated eyes only).

related to the NGF increase observed in serum after topical administration (Fig. 3).

The pharmacokinetic study of topical NGF distribution demonstrated increased levels in the retina, optic nerve, and sclera 2 hours after treatment (200 μg/mL). These achieved peak levels after 6 hours and returned toward baseline by 24 hours (Fig. 4). Forty-eight hours after treatment, NGF levels in the retina, optic nerve, and sclera of topical-NGF–treated eyes had returned to basal levels. A similar trend was observed in ocular tissues of contralateral eyes (Fig. 4). The lens was the only ocular tissue that showed no change in NGF levels after topical treatment. Increases in NGF were not associated with significant changes of NGF mRNA levels in any ocular tissues at any concentration or time point (data not showed).

NGF treatment affected BDNF protein and mRNA levels in the retina of both treated and contralateral eyes (Fig. 5). All NGF doses induced an approximate threefold increase of basal BDNF protein levels, a significant increase compared with both basal and contralateral eyes ($P < 0.005$; Fig 5A). Compared with basal values, a lower but significant increase in BDNF protein was also detected in contralateral eyes of rats receiving the 200 and 500 μg/mL dose of topical NGF (Fig. 5A). Increased levels of BDNF mRNA were also detected in the retinas of NGF-treated eyes at all concentrations used ($P < 0.01$, compared with basal and contralateral eyes). In contralateral eyes, BDNF mRNA levels were affected only after treatment with 200 μg/mL NGF (Fig. 5B).

DISCUSSION

This animal study demonstrated by autoradiography, biochemical and molecular analysis that one topical dose of NGF reached the retina and optic nerve. Because NGF is a high-molecular-weight protein, it was unknown whether it could reach the posterior segment by crossing the ocular surface. There are two routes by which drugs can be absorbed from the ocular surface to reach the posterior segment: through the cornea and through the conjunctiva.[17] High molecular mass drugs of up to 150 kDa do not generally cross the corneal epithelium, but may reach the posterior segment directly by crossing the conjunctiva, sclera, choroid, choriocapillaris, and retinal pigment epithelium, or indirectly by traveling through the retrobulbar space to the optic nerve.[17]

Biologically active NGF (molecular mass, 26 kDa, also called βNGF) was used in this study.[13] Autoradiography demonstrated a considerable presence of radiolabeled NGF in the sclera, but not in the cornea, 2 hours after topical NGF administration (data not shown). ELISA confirmed the increased NGF levels in the sclera. These findings indicate that the increase in NGF in the retina may derive from a direct passage of this protein through the conjunctiva and sclera. Indeed, the rapid and marked uptake of radiolabeled NGF by the optic nerve may have been due to passage through the retrobulbar space as well as to systemic absorption. After topical administration of NGF to one eye, levels peaked in the serum as well as the retina, optic nerve and sclera of the contralateral, untreated eye, strongly indicating that NGF passes through the blood–ocular barrier. In addition, the increased uptake of NGF by RGCs 6 hours after administration provides evidence of the retrotransport of NGF by the optic nerve to the RGCs, as suggested by previously reported data.[8] These time- and dose-dependent increases in NGF in the retina and optic nerve were not associated with increased NGF mRNA synthesis, indicating that exogenous versus endogenous NGF was responsible for the increase observed.

As reported in other studies, enhanced NGF availability is crucial to the survival of RGC and is responsible for functional recovery from ocular ischemia and hypertension.[9–11] Indeed, NGF counteracts photoreceptor degeneration in animal models of retinitis pigmentosa and modulates the optic neuropathy associated with multiple sclerosis.[18–20] In vitro and in vivo studies have demonstrated that NGF exerts neuroprotective effects against apoptosis and glutamate exotoxicity in several neural cells types, including RGCs, and promotes neural plasticity and axonal regeneration.[2,21–24] Moreover, the biological activity of NGF is augmented by its ability to stimulate the production of other growth factors, including BDNF.[2,16,25–28]

In the present study, an increase in BDNF mRNA and protein was observed in rat retinas after NGF treatment. However, this BDNF increase was not NGF dose dependent, suggesting that BDNF expression is triggered by complex mechanisms only partially related to the increase of retinal NGF. This NGF-driven BDNF synthesis[16,29] may contribute to or enhance the neuroprotective effects of this protein. Several reports demonstrated a key role of BDNF in regulating the survival and function of RGCs after optic nerve crush and hypertensive injury.[30–33]

In conclusion, these findings indicate that NGF applied topically to the eye reaches, and is pharmacologically active in, the posterior segment. This protein is a promising candidate as a neuroprotective agent in ocular diseases characterized by retinal cell apoptosis (glaucoma, ischemic and traumatic optic neuropathy, and optic neuritis) and in forms of retinal degeneration.[34–36] Further investigation into the effects of topical NGF may lead to the development of an adjunctive therapy in

glaucoma and other diseases that may significantly reduce neuron death and nerve loss.

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Experimental and clinical evidence of neuroprotection by nerve growth factor eye drops: Implications for glaucoma

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Elevated intraocular pressure (IOP) in glaucoma causes loss of retinal ganglion cells (RGCs) and damage to the optic nerve. Although IOP is controlled pharmacologically, no treatment is available to restore retinal and optic nerve function. We evaluated the effects of NGF eye drops in a rat model of glaucoma. We also treated 3 patients with progressive visual field defects despite IOP control. Glaucoma was induced in rats through injection of hypertonic saline into the episcleral vein. Initially, 2 doses of NGF (100 and 200 μg/mL) were tested on 24 rats, and the higher dose was found to be more effective. Glaucoma was then induced in an additional 36 rats: half untreated and half treated with 200 μg/mL NGF QID for 7 weeks. Apoptosis/survival of RGCs was evaluated by histological, biochemical, and molecular analysis. Three patients with advanced glaucoma underwent psychofunctional and electrofunctional tests at baseline, after 3 months of NGF eye drops, and after 3 months of follow-up. Seven weeks of elevated IOP caused RGC degeneration resulting in 40% cell death. Significantly less RGC loss was observed with NGF treatment (2,530 ± 121 vs. 1,850 ± 156 RGCs/mm²) associated with inhibition of cell death by apoptosis. Patients treated with NGF demonstrated long lasting improvements in visual field, optic nerve function, contrast sensitivity, and visual acuity. NGF exerted neuroprotective effects, inhibiting apoptosis of RGCs in animals with glaucoma. In 3 patients with advanced glaucoma, treatment with topical NGF improved all parameters of visual function. These results may open therapeutic perspectives for glaucoma and other neurodegenerative diseases.

NGF | optic nerve | retina

Glaucoma is the leading cause of irreversible blindness in the world (1). This chronic and progressive optic neuropathy is characterized by loss of axons of the retinal ganglion cells (RGC) that constitute the optic nerve (2). Elevated intraocular pressure (IOP) is the primary risk factor for glaucoma, responsible for long-term damage to the optic nerve (3). Patients with glaucoma typically lose their visual field and become blind if untreated. Reduction of IOP, the only modifiable causative factor, slows the onset and progression of the disease, yet no actual treatment is available to restore optic nerve damage (4).

Neuroprotection has gained substantial interest in recent years as a therapeutic approach to preventing neuronal degeneration and loss of function in glaucoma (4). Neuroprotective therapies currently under investigation to restore retinal/neural function include memantine, neurotrophins, erythropoietin, reactive oxygen species scavengers, and even vaccine therapies (4–6). Nevertheless, results of these randomized clinical trials have so far been inadequate.

Nerve growth factor (NGF) is an endogenous neurotrophin that exerts trophic and differentiating activity on neurons of the central and peripheral nervous systems with protective and/or regenerative effects observed in degenerative diseases or following injury (7–9). Intracerebral administration of NGF has been shown to be beneficial in Parkinson's and Alzheimer's patients (10–12), and intraocular administration of NGF in animal models has been shown to inhibit RGC degeneration after mechanical, ischemic or hypertensive injury (13–15). NGF applied topically to the eye has also been shown to restore sensory nerve function to the ocular surface of patients with neurotrophic keratitis (16). Interestingly, absorption studies have demonstrated that topical ocular NGF reaches the retina, optic nerve, and brain in animals (17, 18).

In the present study, we demonstrate that topical application of exogenous murine NGF to the eye prevents RGC degeneration in an experimental rat model of glaucoma. Based on these findings, we used the same dosage regimen to treat 3 patients with rapid and progressive visual field loss despite successful treatment of ocular hypertension.

Results

Effects of Episcleral Venous Injection of Hypertonic Saline. At baseline time 0, the mean IOP in SD rats was 24.6 ± 2.1 mm Hg and 24.7 ± 2 mm Hg in the control and experimental eyes, respectively. Significant unilateral elevation of IOP was successfully induced in the glaucomatous eyes by saline injection into the episcleral veins (Fig. 1), as shown by mean IOP values measured weekly for 7 weeks starting 1 week after treatment (Fig. 2*A*). Mean IOP in the saline injected eyes was 35.8 ± 3.2 mm Hg, compared to 24.7 ± 2.2 mm Hg in the contralateral, sham operated eyes ($P < 0.01$).

Effects of Elevated IOP on RGC. Histological evaluation indicated that compared to normal healthy retinas (Fig. 2*B*), 7 weeks of chronically elevated IOP induced approximately a 40% decrease in the number of RGCs (Fig. 2*C*; 1,861 ± 106 RGC/mm² vs. 3,155 ± 98 RGC/mm², respectively, $P < 0.01$, Fig. 2*D*). To assess whether the reduced RGC number was due to cell death through apoptosis, biomarkers involved in cell death and cell survival were studied. As shown in Fig. 3 *A–C*, anti-TUNEL staining, a biomarker for apoptotic cell death, was greater in RGCs of rats with elevated IOP compared to healthy controls (6 ± 0.9 vs. 0.4 ± 0.3 per mm², $P < 0.01$). Moreover, molecular analysis of Bcl-2, a biomarker of cell survival, and Bax, a marker of cell death, demonstrated a lower mRNA Bcl-2/Bax ratio in the experimentally induced, untreated glaucomatous eyes (Fig. 3*D*, Ct values inversely correlated with mRNA expression values). The results of western blot analysis reported in Fig. 3*E* confirmed the molecular data indicating a lower Bcl-2/Bax protein ratio in glaucomatous eyes.

Effects of Topical NGF in the Animal Model of Glaucoma. Preliminary experiments comparing 100 and 200 μg/mL NGF eye drops showed

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The authors declare no conflict of interest.

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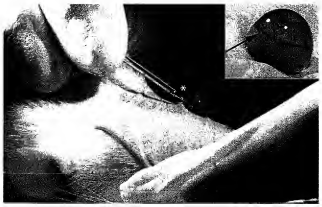

Fig. 1. Glaucoma was induced in adult SD rats by single injection of 50 μL hypertonic saline solution (1.75M NaCl) into the superior episcleral vein. Once the rats were anesthetized, episcleral veins were isolated (arrow) under a led microscope and injections were performed using custom-made microneedle glass syringes (asterisk).

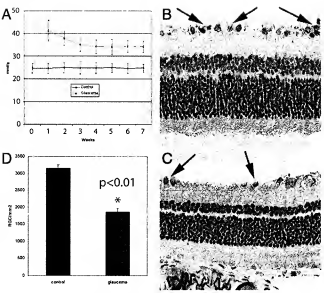

Fig. 2. Measurement of intraocular pressure (IOP) demonstrated significant increases ($P < 0.01$) in rats that received episcleral injection with hypertonic saline solution (*A*). Hematoxylin/eosin staining of normal (*B*) and glaucomatous (*C*) retinas showed a significantly lower ($P < 0.01$) number of RGCs (arrows) in glaucomatous eyes (*D*).

a significantly higher biologic effect of 200 μg/mL NGF eye drops in protecting RGC loss in retinal sections from glaucomatous rat eyes, as demonstrated by E/E staining (2,145 ± 102 vs. 2,623 ± 138 RGCs/mm^2, respectively, $P < 0.05$). Thus, in subsequent experiments, the 200-μg/mL concentration was used.

Histological analysis showed that topical ophthalmic administration 4 times daily with 200 μg/mL NGF for 7 consecutive weeks protected RGCs of rats with glaucoma (Fig. 4 *A–H*). Specifically, glaucomatous eyes treated with NGF had significantly more RCGs than a parallel group of glaucomatous eyes not treated with NGF (2,530 ± 121 vs. 1,850 ± 156 RGCs/mm^2, $P < 0.01$). NGF-treated glaucomatous eyes also had significantly less anti-TUNEL staining of RGCs (1.2 ± 0.6 vs. 6 ± 0.9 per mm^2, $P < 0.01$), and greater RGC survival, as shown by the significantly higher Bcl-2/Bax ratio (Fig. 4*G*, Ct values inversely correlated with mRNA expression values). The results of western blot protein analysis confirmed the significantly higher Bcl-2/Bax ratio in NGF-treated glaucomatous eyes, indicating greater RGC survival compared to the RGCs of untreated glaucomatous eyes.

No statistically significant differences were observed between NGF-treated glaucomatous eyes and control eyes in TUNEL staining (1.2 ± 0.6 vs. 0.4 ± 0.3 per mm^2) and Bcl-2/Bax ratio. Although RGC cell count showed a protective effect of NGF in glaucomatous eyes, a significantly higher number of RGCs was still observed in control eyes as compared to NGF-treated glaucomatous eyes (3,155 ± 98 vs. 2,530 ± 121, $P < 0.05$).

Effects of Topical NGF in Patients with Glaucoma. All 3 patients treated with 200 μg/mL NGF showed improvements in psychofunctional and electrofunctional parameters after 3 months of treatment. This effect was sustained even after a subsequent 3 months without NGF therapy. The patients with glaucoma had severe dysfunction of the innermost retinal layers and a delay of neural conduction along the postretinal visual pathways, as indicated by PERG P50 and VEP P100 values with longer time-to-peaks and longer RCT and PERG P50-N95 with reduced amplitudes with respect to control data (19). A progressive improvement of inner retinal layer function and postretinal neural conduction was observed during NGF treatment. This enhanced neuronal function was then maintained even 3 months after discontinuation of NGF treatment (Table 1 and Fig. 5).

These electrophysiological changes were accompanied by improvements in clinical parameters as well. Visual field mean defects (MD) improved from 0% to 5% in all patients by the end of NGF treatment (Table 1 and Fig. 6) and a further 1% to 15.8% 3 months after NGF discontinuation.

Contrast sensitivity at 12 cyc/deg in Patient 1 improved from 0.91 (baseline) to 1.080 (15.7%) (end of NGF treatment); in Patient 2, from 0.91 to 1.080 (15.7%), and in Patient 3 from 1.080 to 1.250 (13.6%). These values remained unchanged 3 months after discontinuation of NGF treatment (Table 1).

The best corrected visual acuity in Patient 1 improved from 0.4 to 0.7 (42.8%), in Patient 2, from 0.4 to 0.8 (50%), and in Patient 3 from 0.5 to 0.8 (37.5%). These visual acuity values remained unchanged 3 months after discontinuation of NGF treatment (Table 1).

No side effects were observed during NGF treatment and during the follow-up period, with the exception of a transient (1 week) burning sensation in 1 patient.

Discussion

This study demonstrated that murine NGF administered topically to the eye rescued RGCs from apoptosis in rats. We used a well-characterized experimental model of glaucoma, in which a single injection of hypertonic saline into the episcleral veins of rat eyes induced chronic elevation of IOP, optic-nerve degeneration, and selective RGC loss by apoptosis, the sum effects of which resemble human glaucoma (20–22). The beneficial effect of NGF on RGC survival was demonstrated to be due to inhibition of apoptosis, as shown by the reduction in TUNEL RGC immunostaining and the greater retinal Bcl-2/Bax ratio.

It is known that RGCs express NGF receptor (TrkA) and that NGF binding to TrkA up-regulates Bcl-2 protein, which protects cells from apoptosis by preventing caspase activation (21, 23). Furthermore, intravitreal NGF delivery to the retina and optic nerve is crucial to the survival of RGCs and NGF is known to be responsible for functional recovery of the retina following ocular ischemia and hypertension in animal models (13–15). Lastly, an ophthalmic solution of NGF administered topically to the ocular surface has been shown to reach the retina and optic nerve where it is biologically active (17).

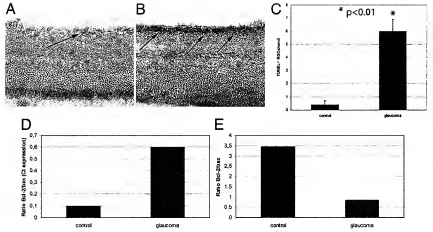

Fig. 3. Anti-TUNEL immunostaining of RGCs (arrows) in normal (*A*) and glaucomatous (*B*) eyes showed a significantly greater ($P < 0.01$) number of apoptotic RGCs in glaucomatous eyes (*C*). Molecular analysis of glaucomatous compared to normal, healthy retinas showed greater mRNA expression of Bax (a biomarker of cell apoptosis) and lower expression of Bcl2 (a biomarker for cell survival), as illustrated by the Bcl-2/Bax ratio (*D*). The results of western blot protein analysis confirmed the significantly lower Bcl-2/Bax ratio in glaucomatous retinas (*E*).

Three months of topical, ocular NGF treatment in 3 patients with advanced glaucoma at risk of vision loss resulted in long-lasting improvement in visual field, contrast sensitivity, and best corrected visual acuity. Despite successful IOP control by medical therapy, these patients had progressive visual field defects and severe abnormalities in PERG and VEP responses that indicated dysfunction of the innermost retinal layers, delay in visual cortical responses, and delay in neural conduction along postretinal visual pathways (24, 25). In glaucoma, up to 20% of patients show progression of visual field defects with RGC and optic nerve degeneration despite successful management of ocular hypertension (26). In fact, elevated IOP is thought to be only the *primum movens* that triggers a cascade of events leading to optic nerve damage (4). An approach that would vastly improve the treatment of this challenging disease would involve neuroprotection with exogenous neurotrophic factors (6, 27).

PERG and VEP amplitudes and times-to-peak were the first electrofunctional parameters improved in our patients, suggesting a functional recovery of RGCs and an improvement of neural conduction along the postretinal visual pathways. The observed increase in CSV at 12 cyc/deg further supports the efficacy of NGF treatment on RGC (28). These effects are in line with the crucial role of neurotrophins in modulating RGC function and visual cortical neuronal activity reflected by receptive field size, orientation selectivity, visual acuity, response latency, and habituation (29–31).

NGF treatment also improved mean visual field defects in 2 patients (patient 1 and 2), and stabilized the defect in the third patient. Improvement of visual field persisted 90 days after discontinuation of treatment, indicating that changes induced by NGF had a prolonged duration. Two patients were actually followed up after 18 months, at which time improvements in visual field were still stable. This "long-term" NGF effect may be related not only to a protective activity against neural apoptosis, but also to the formation of new neural pathways, since it is known that NGF promotes neural plasticity and axonal regeneration (8, 32–34). In fact, NGF

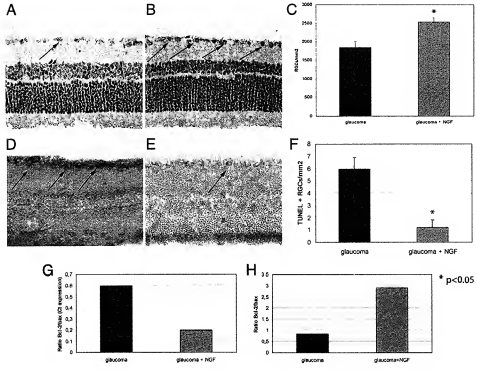

Fig. 4. Hematoxylin/eosin staining of retinas from untreated (*A*) and NGF-treated (*B*) glaucomatous eyes showed significantly less ($P < 0.05$) loss of RGCs (arrows) in animals that received 200 μg/mL NGF eye drops (*C*). Anti-TUNEL immunostaining of retinas from untreated (*D*) and NGF-treated (*E*) glaucomatous eyes showed significantly less ($P < 0.05$) apoptotic RGCs (arrows) in animals that received NGF (*F*). Molecular analysis showed significantly lower mRNA expression of Bax (a biomarker of cell apoptosis) associated with greater expression of Bcl2 (a biomarker for cell survival), as illustrated by the Bcl-2/Bax ratio (*G*), in glaucomatous eyes treated with NGF compared to untreated glaucomatous eyes. Western blot analysis of Bcl and 2/Bax (*H*) confirmed this protective effect of NGF.

Table 1. Effects of NGF treatment in 3 patients affected by advanced glaucoma

	Age, Sex	IOP, mmHg	Visual field, MD	PERG P50 latency, ms	PERG P50-N95 amplitude, μV	VEP P100 latency, ms	VEP N75–100 amplitude, μV	Retinocortical time, ms	CSV 3°	CSV 6°	CSV 12°	CSV 18°	Visual
						Baseline							
Patient 1	74, M	15	−32.90	63	1.0	167	1.3	104	1.085	1.210	1.080	0.470	0.40
Patient 2	59, M	15	−33.90	69	0.8	145	0.7	76	1.085	1.290	0.910	0.640	0.40
Patient 3	82, F	18	−34.27	60	0.8	179	1.4	119	1.160	1.210	0.910	0.640	0.50
						At 1 month of NGF treatment							
Patient 1		17	−33.14	57	1.5	150	2.9	93	1.085	1.290	0.910	0.640	0.40
Patient 2		15	−33.15	60	1.2	122	2.4	62	1.160	1.210	0.910	0.640	0.40
Patient 3		15	−34.40	63	1.1	168	1.5	105	1.085	1.210	1.080	0.470	0.50
						At 3 months of NGF treatment							
Patient 1		15	−31.50	52	1.6	145	5.2	93	1.010	1.290	0.910	0.640	0.40
Patient 2		12	−32.10	67	1.5	109	2.8	42	1.850	1.540	0.910	0.710	0.70
Patient 3		17	−34.30	59	1.4	174	2.6	115	1.010	1.210	0.910	0.470	0.60
						At 3 months of NGF treatment discontinuation							
Patient 1		14	−27.70	52	1.6	145	5.2	93	1.630	1.850	1.080	0.640	0.70
Patient 2		12	−29.20	67	1.5	109	2.8	42	2.000	1.530	1.080	0.800	0.80
Patient 3		16	−33.90	59	1.4	174	2.6	115	1.010	1.210	1.250	0.470	0.80

acts on numerous levels to promote neuronal recovery following ischemic and chemical injuries: through a neosynaptogenetic mechanism, by directly affecting precursor cells and/or by induction of other growth factors, including BDNF (8, 35–37). These multiple activities may cause the progressive improvement in visual acuity observed in our 3 patients during and after NGF treatment (Table 1).

The neuroprotective effects of NGF in glaucoma demonstrated in this study, together with the recently gained knowledge of NGF's ability to reach the brain when topically administered to the eye, allude to exciting possibilities for the treatment of neurodegenerative diseases of the central nervous system (18, 38). A major challenge in treating neurodegenerative disorders such as Alzheimer's disease has been the difficulty of delivering neurotrophic factors across the blood-brain barrier (8). This obstacle might be overcome by ophthalmic topical NGF treatment, and absorption and diffusion studies following this premise should be undertaken.

Many similarities between glaucoma and Alzheimer's disease go far beyond the challenges encountered in their treatment: (*i*) RGCs die by apoptosis in glaucoma through activation of specific caspases, which are also activated in Alzheimer's; (*ii*) caspase activation with cleavage of APP has been shown to up-regulate amyloid-beta production in Alzheimer's and in animal models of glaucoma; (*iii*) age-related mitochondrial dysfunction plays a key role in the etiology of both neurodegenerative disorders; (*iv*) elevated glutamate and nitric oxide synthase up-regulation with reactive oxygen species formation have been implicated in both glaucoma and Alzheimer's neurotoxicity; and (*v*) glutamate toxicity is involved in both glaucoma and Alzheimer's synaptic dysfunction (39, 40). All of these similarities have led glaucoma to be dubbed the "ocular Alzheimer's disease" (39). The obvious benefit to this likeness is the combining of forces in identifying new strategies to treat either disease.

In summary, this study indicates that topical NGF treatment may be an effective adjunct therapy for glaucoma, reducing neuron death and nerve loss. These encouraging results merit further investigation of topical NGF in controlled clinical trials in glaucoma and other forms of neurodegenerative disorders.

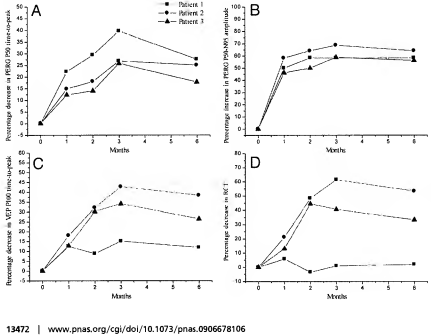

Fig. 5. Results of NGF eye drop treatment in 3 glaucomatous patients. Presented are relative changes in the electrophysiological parameters that reflected function of the innermost retinal layer (Panels *A* and *B*: pattern electroretinogram: PERG P50 time-to-peak and P50-N95 amplitude), the bioelectric visual cortical response (Panel *C*: visual evoked potential: VEP P100 time-to-peak) and neural conduction along the postretinal visual pathways (Panel *D*: retinocortical time: RCT) observed after 30, 60, and 90 days of NGF treatment and after another 90 days of follow-up (time 1, 2, 3, and 6) with respect to the baseline condition (time 0). The relative changes are expressed as percentage increase in amplitude or percentage decrease in time-to-peak from baseline. Percentage increases in PERG P50-N95 amplitude and percentage decreases in PERG P50 time-to-peak indicated a reduction in ganglion cell dysfunction after NFG treatment. Percentage decreases in VEP P100 time-to-peak and percentage decreases in RCT indicated a reduction of the neural conduction delay along visual pathways after NFG treatment.

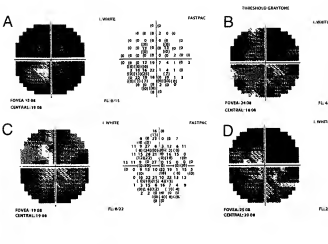

Fig. 6. A representative visual field illustrate changes from baseline (*A*) to 1 month of NGF treatment (*B*), to 3 months of NGF treatment (*C*) and to 3 months after discontinuation of NGF (*D*) in a patient affected by advanced glaucoma.

Materials and Methods

For this study, we used pathogen-free, adult male Sprague-Dawley (SD) rats (n = 78) maintained on a 12-h light-dark cycle and provided with food and water ad libitum. All procedures regarding housing, care and experimental procedures were carried out following the guidelines of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, International law (EEC council directive 86/609, OJ L 358, 1, December 12, 1987), and the Italian National Research Council's Ethical Commission on Animal Experimentation (1992).

Preliminary histological studies aimed to identify the best NGF concentration were performed on 24 rats: 6 untreated normal rats (control group), 6 untreated rats with glaucoma, 6 100 μg/mL NGF-treated rats with glaucoma, and 6 200 μg/mL NGF-treated rats with glaucoma. NGF was administered 4 times daily for 7 consecutive weeks. Based on these results, a second set of experiments was performed on an additional 54 rats: 18 normal controls, 18 NGF-treated (200 μg/mL) rats with glaucoma and 18 untreated rats with glaucoma. NGF was administered with the same dosage regimen, 4 times daily for 7 weeks.

Animal Model of Glaucoma. Glaucoma was induced as described by Morrison et al. (20) Briefly, was injected once into the superior episcleral vein of 1 eye (Fig. 1), indicated as the ipsilateral glaucomatous eye, while the contralateral eye served as a sham, non-glaucomatous control eye. Glaucoma was defined as a significant loss of RGCs by apoptosis (20, 21). Rats were housed in single cages in a constant low-light environment (40–90 lux) to minimize IOP circadian oscillations and treated as indicated below.

IOP was measured weekly with a TonoPen XL tonometer (Mentor) under topical anesthesia and the values recorded were the mean of 10 valid measurements, expressed as TonPen readings. Mean changes were then calculated for each eye ± the standard deviation of the mean (SD). NGF treatment was initiated immediately at time 0, and results were compared among the 3 parallel groups: healthy eyes, glaucomatous eyes, and glaucomatous eyes treated with NGF.

Histological Evaluation. Rats were euthanized with an overdose of Nembutal after 7 weeks. Eyes were removed and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for 24 h. For light histological analysis of the retina, eyes were fixed in Bouin's fluid for 1 week, and then immersed for 3 days in phosphate buffer containing 20% sucrose, pH 7.4. Retinal sections 20 μm in width were cut with a cryostat at 4 °C, and stained with haematoxylin-eosin. Using ImageJ image processing and analysis software, RGCs were counted in a masked fashion in 4 quadrants of the retinal sections approximately 2 mm from the center of the optic disc. Counts were taken from comparable areas under a Zeiss Microscope at 400× magnification. The results were averaged and converted to cells per mm^2.

Analysis of Cell Death. For the determination of RGC death and survival, sections of the retina were immunostained with TUNEL (TdT-dUTP Terminal Nick-End), a marker of apoptotic cell death, combined with western blot and molecular analyses for Bcl-2 and Bax. Briefly, retinal sections were first incubated in a blocking solution (3% H_2O_2 in methanol) for 10 min at 15–25 °C, incubated in a permeabilization solution containing 1× PBS 0.1% Triton X-100 for 2 min at 4 °C, and then labeled with an in situ cell death detection kit (Roche Diagnostic, Boheringer) according to the manufacturer's instructions. For TUNEL-positive cells, DNA strand breaks were labeled and visualized with 0.4% DAB-H_2O_2. TUNEL-positive cells with nuclear condensation or fragmentation were considered as apoptotic cells.

For Bcl-2 and Bax western blot experiments, protein concentrations were determined using the Micro BCA protein assay kit. After determination of protein concentrations, equivalent amounts of retinal lysates (50 μg) were denatured in sample buffer (final concentration of 2% SDS, 10% glycerol, and 2% 2-mercaptoethanol, pH 6.8) and electrophoresed through 10% denaturing polyacrylamide gels. Following SDS polyacrylamide gel electrophoresis, samples were transferred electrophoretically to nitrocellulose membranes in transfer buffer. Membranes were blocked for 1 h in 1× TBS0.1% Tween-20 with 5% defatted milk powder. Anti-Bcl-2 and anti-Bax primary antibodies (Santa Cruz Biotechnology) were incubated with the appropriate membranes at a dilution of 1:500 overnight. The GAPDH primary antibody (Sigma) was used at a dilution of 1:5,000. Appropriate HRP-conjugated secondary antibodies, all diluted to 1:2,500, were incubated with the membranes for 1 h. After incubation with secondary antibody, membranes were washed 3 times in 1× TBS (pH 7.4) with 1% Tween-20, and then developed using chemiluminescence. Images were digitalized in a Kodak Imager Station and bands were subjected to densitometric analysis using 1D Kodak software.

Molecular Analysis/Real-time PCR. Bcl-2 and Bax mRNA were measured in rat retinas (average 0.010 mg wet weight for each sample). Tissues were pretreated with proteinase K (20 mg/mL, Finnzyme) in HIRT buffer at 56 °C/3 h, and total RNA was extracted from samples using the Puregene RNA purification kit (Gentra Systems). The resulting total RNA was re-suspended in 25 μL diethyl pyrocarbonate-treated water (ICN) and treated with RNase-Free DNase I to eliminate any genomic DNA contamination according to the supplier's protocol (2 U/μL Turbo DNA free kit AM-1907; Ambion Ltd.). Total RNA samples were checked for both RNA quantity (Nanodrop; Celbio), purity (>1.6) and absence of any RNA degradation (RIN ~8). Equivalent amounts of RNA (3 μg) per sample were used as a template in normalized cDNA synthesis. Reverse transcription was performed according to the standardized Mu-MLV protocol (final volume reaction of 20 μL using 50 pM oligo dT-primer, 1 mM dNTP mix, and 200 U reverse transcriptase; Mu-MLV, F-605L; Finnzyme) in a PTC-100 programmable thermocycler (MJ Research). The resulting cDNA was amplified using the SYBR Green PCR core reagent kit (Applied Biosystems) and an Opticon2 MJ Research system (MJ Research). The reaction contained 10 μL SYBR reagent, 3 μL cDNA (for the target) or 1 μL cDNA (for the referring gene), and 20 nM primers in a 20-μL final volume. The temperature profile included initial denaturation at 95 °C/15 min, followed by 35–47 cycles of denaturation at 95 °C/30 s, annealing at 55–60 °C/25 s (the annealing time depended on the primer's Tm), elongation at 72 °C/30 s, fluorescence monitoring at 60–90 °C, and further incubation at 72 °C/5 m. Specific previously published primers for Bcl2 were used for this study (21). Primer specificities were further confirmed by the single melting curves obtained during each amplification. Negative controls (without template) were produced for each run. Experiments were performed in duplicate for each data point. Quantitative values were obtained from the threshold cycle value (Ct), which is the point where a significant increase of fluorescence is first detected. According to the REST© software, results are expressed as N-fold difference (increase or decrease) in target gene expression. Lastly, ratios between Bcl-2/Bax were calculated according to the single Ct values.

NGF Eye Drop Preparation and Treatment. NGF was obtained from murine salivary glands as previously described, following the Bocchini and Angelett method (41). Briefly, gel filtration at pH 7.5 was performed on the aqueous gland extract of adult mice, followed by dialysis at pH 5.0 and fractioning by cellulose-chromatography. In the present study, the biologically active form of highly

purified murine NGF weighing 26 kDa was used, dissolved in a sterile 0.9% NaCl solution at 2 different concentrations (100 and 200 μg/mL) (17).

Selection of Patients. Three patients (69 $\pm$ 6 years old, 2 males and 1 female) affected by advanced and progressive glaucoma (disease duration 21 $\pm$ 9 years), with impending risk of vision loss, despite good pharmacological control (timolol 0.5% and pilocarpine 2% in a fixed combination BID and latanoprost QID) of intraocular pressure (measured by applanation tonometry) were included in the study. Advanced glaucoma was defined by the following functional criteria: a mean deviation less than -24 dB, the presence of only a central or temporal island remaining in the visual field gray scale (42); and optic disk rim deterioration as an additional morphological criterion (43).

Treatment Regimen in Patients with Glaucoma. Based on the dosage regimen used in the animal model, the 3 patients were treated topically with 1 drop ($\approx$50 μL) of highly purified murine NGF, 200 μg/mL, instilled into the conjunctival fornix of 1 eye only 4 times daily for 3 months.

The tenets of the Declaration of Helsinki were followed in this study. Informed consent was obtained from the subjects after explanation of the nature and possible consequences of the study. All patients were at imminent risk of irreversible and complete vision loss for uncontrolled progression of glaucoma despite adequate IOP control and were, therefore, treated on a compassionate basis.

Electrofunctional and Psychosensorial Evaluation of Patients. Patients were evaluated at baseline, every month during treatment and 3 months post-therapy by complete ocular examination including visual acuity, tonometry, optic disk photography, contrast sensitivity (CSV-1000, Vector Vision), visual field (Humphrey, program 10/2), and electrofunctional tests (Pattern Electroretinogram, PERG, Visual Evoked Potentials, VEPs). Static perimetry was also performed and repeated 3 times using a Humphrey field analyzer (model 740, central 10–2 achromatic full threshold strategy, showing fixation losses, false positive rate, and false negative rates each less than 20%, and numeric loss of sensitivity). The mean defect (MD) defined the mean obtained in all tested points, considering the increasing scatter of sensitivity values with respect to the data obtained in normal subjects according to eccentricity, and therefore indicating the severity of global damage (44). We used the central 10° with a finer grid pattern to improve resolution of the remaining visual field and to reduce testing time.

Foveal contrast sensitivity was tested using a commercially available chart (CSV1000; Vector Vision). At the testing distance of 8 feet, the translucent chart presents 4 spatial frequencies, each on a separate row; 3, 6, 12, and 18 cyc/deg. According to the Pomerance and Evans procedure (28), the sensitivity threshold was measured twice, allowing only a few seconds between measurements. The second measurements were considered for analysis. The test-retest variability was consistent with that previously reported.

Simultaneous recordings of VEPs and PERGs were assessed using a previously published method (19). Transient VEP was characterized by 3 peaks that appeared after 75, 100, and 145 ms and had negative (N75), positive (P100), and negative (N145) polarity, respectively. The transient PERG was characterized by 3 peaks that appeared after 35, 50, and 95 ms and had negative (N35), positive (P50), and negative (N95) polarity, respectively. Amplitudes (in mV) and time-to-peaks (in ms) were measured. Simultaneous recordings of VEPs and PERGs identified an index of neural conduction along the postretinal visual pathways, defined as retinocortical time (RCT, the difference between the VEP P100 and the PERG P50 time-to-peak).

Data Analysis. Statistical analysis was performed using the SuperANOVA package for Macintosh (Abacus Concepts Inc.) and the Tukey-Kramer comparison; a P value of less than 0.05 was considered statistically significant. Animal data of parallel control groups were evaluated and compared at endpoint. While the group of 3 patients was too small to elaborate statistically, the data presented are individual changes over time from baseline.

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Ocular Application of Nerve Growth Factor Protects Degenerating Retinal Ganglion Cells in a Rat Model of Glaucoma

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Purpose: Elevated intraocular pressure is a crucial pathologic event for the development of glaucoma (GL). We have reported that nerve growth factor (NGF) reaches retinal cells and the optic nerve (ON) when applied to the eye. Whether ocular application of NGF prevents or reduces damage to retinal ganglion cell (RGC) is not known.

Methods: GL was induced in adult rats by the injection of hypertonic saline into the episcleral vein of the right eye and the left eye used as control. Rats were then treated daily with ocular application of NGF or vehicle solution for 7 weeks. Retinal and ON tissues were then used for structural, immunohistochemical, and biochemical studies.

Results: The injection of hypertonic saline into the episcleral vein led to progressive degeneration of RGCs, with the loss of nearly 40% of these cells after 7 weeks of treatment. This cellular loss is associated with the downregulation of NGF and NGF-receptor expression in the retina and ON of the glaucomatous eye and ocular treatment with NGF significantly reduced the deficit induced by GL.

Conclusions: These findings indicate that NGF can exert protective action on RGC degeneration occurring in glaucomatous retina. We suggest that ocular NGF treatment might be a suitable pharmacologic approach to investigate protective mechanisms of degenerating RGCs.

Key Words: glaucoma (GL), intraocular pressure (IOP), NGF-receptor, neurotrophins, retinal ganglion cell (RGC)

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Glaucoma (GL) is the second leading cause of blindness worldwide and the prevalence of this disease is expected to grow as the population ages.[1] GL is a disease characterized by progressive death of the retinal ganglion cells (RGCs) leads to optic nerve (ON) degeneration and vision loss. However, although the elevated intraocular pressure (EIOP) is considered a primary cause of the visual deficit, it is known that some patients still experience visual loss after lowering the IOP and others with GL and ON degeneration can have normal IOP.[2–4] Despite these evidence, a major therapeutic aim is to facilitate the survival of RGCs through pharmacologic or surgical reduction of IOP.[5–8] These and other observations have led to the hypothesis that degeneration of retinal cells may result from a secondary insult induced by alterations in the neuronal environment, such as changes in neurotransmitters, influx of calcium into the cells, formation of free radicals, or depletion of neurotrophic factors.[5–8]

Nerve growth factor (NGF) is the first identified and best-characterized neuroprotective molecule, acting upon and produced by a number of cells within and outside the nervous system.[9–11] The biologic activity of NGF is regulated by 2 different types of receptors that are located on the surface of responsive cells: the high-affinity NGF-receptor (TrkA), which belongs to the family of tyrosine kinase receptors, and the low-affinity NGF-receptor (p75), which is a transmembrane glycoprotein that lacks a tyrosine kinase domain.[12,13] There is now a number of studies demonstrating that NGF is able to exert a wide spectrum of effects also on cells of the visual system, playing a crucial role in promoting the survival and growth of retinal cells. Thus, abnormal presence of NGF or its receptors can lead to cell death, not only in the central and peripheral nervous system,[9–11] but also in the visual system in vivo[14–22] and in vitro.[23,24] NGF promotes the functional recovery of RGCs in an animal model of ocular ischemia,[16–21] reduces retinal cell damage induced by intraocular hypertension,[19] and delays retinal cell degeneration in rodents with retinitis pigmentosa.[17] The functional role of NGF on retinal cells is supported by studies showing that intravitreal injection of NGF increases RGC survival and that eye NGF deprivation is known to lead to RGC death.[19] One primary obstacle to clinical testing of NGF for retinal disorders is, however, the lack of an efficient, noninvasive means to deliver this factor in the posterior segment of the eye to the target cells. We have recently reported that intraocular administration of radiolabeled NGF binds to RGCs and ON axons[25] and that conjunctival applied NGF reaches retinal cells and brain NGF-receptive neurons.[26,27] These observations suggested to investigate whether eye application of NGF protects retinal cells degeneration in a rat model of GL induced by EIOP in adult rats. The results of

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these studies indicate that GL induced in rats with EIOP reduces significantly the presence of NGF and the number of RGCs and ON axons, whereas ocular NGF administration significantly protects these degenerative events.

METHODS

Animals and Surgical Procedures

GL was induced in pathogen-free adult male Sprague-Dawley rats (n = 94), after anesthesia (ketamine 100 mg/mL; xylazine 20 mg/mL; and acepromazine 10 mg/mL). Briefly, as described by Morrison et al,[28] after a lateral canthotomy, a small plastic ring was fitted around the globe, straddling the equator and oriented to provide unobstructed passage for one radial aqueous vein in the superior quadrant of the eye, and then rats received 50 μL sterile hypertonic saline solution (1.75 M NaCl) into the superior episcleral vein of the right eye. All animals were maintained in a 12 hours (06:00 to 18:00) light: dark cycle. The right eye was indicated as the glaucomatous eye and the left eye was indicated as control eye. Operated rats were divided into 2 groups and housed in single cages in a constant low-light environment (40 to 90 lx) to minimize IOP circadian oscillations. IOP was measured weekly with the TonoPen XL tonometer (Medtronic Ophthalmics, Jacksonville, FL) under topical anesthesia. IOP is reported as the mean of 10 valid readings per eye. Mean changes were calculated for each eye ± SEM. The housing, care, and experimental procedures involving the experimental rats were carried out in accordance with the guidelines set by the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research and by local Ethical Commission on Animal Experimentation.

Animal Treatments

NGF was purchased from Biolab (Rome, Italy) or purified from adult male mouse submandibular glands following the method of Bocchini and Angeletti.[29] Purified NGF was dissolved in 0.9% NaCl phospate (200 μg/mL) and kept at 4°C for no longer than 1 week. Under these conditions, both stability and activity remained unchanged. Rats (n = 42) received topical ocular administration of 20 μL vehicle solution, consisting of 0.9% NaCl, twice a day in both the right glaucomatous eye (GLV) and the left non-glaucomatous control eye (CV) for 7 weeks.

A second group of rats (n = 42) received topical ocular administration of 20 μL of this NGF solution (4 μg of purified NGF) 2 times a day for 7 weeks in the right glaucomatous eye (GLNGF) and the left control eye (CNGF). Animals were sacrificed with an overdose of Nembutal at time 0, 1, 3, and 7 weeks after the induction of GL.

Histologic Analysis of the Retina

For light histologic analysis of the retina, 5 eyes with attached ON were removed from 5 different animals of each experimental group (n = 4), fixed in Bouin fluid for 1 week, and then immersed in phosphate-buffered saline (PBS), pH 7.4, containing 20% sucrose for 3 days as described earlier.[26] Coded sections of the eyes were cut at 20 μm with a cryostat (Leica CM 1850 UV, Germany) at −20°C, taking care that the cross sections of each retina were performed in the same orientation. Sections were then stained with hematoxylin-eosin. The number of RGCs were counted under a Zeiss microscope equipped with a 40 × objective. To obtain representative data regarding the number of RGCs, RGC counts were performed on 5 random areas, located approximately 2 mm from the centre of the optic disc, of 6 different sections of each retina. All counts were performed in a masked manner and the results were averaged and converted to cells/mm^2.

Ultrastructural Analysis of the ON

The ON, located 1 mm from the adjacent retina, was dissected out, was fixed in 4% paraformaldehyde in 0.1 M PBS, pH 7.4, for 24 hours and then post-fixed in 1% OsO_4 for 4 hours, dehydrated with ascending ethanol, and then embedded in 812/Spurr's low viscosity resin. ON sections were cut with a Reichert microtome, collected on 200 mesh grids, stained with uranyl-acetate and lead citrate, observed under a Philips 100 electron microscope, and photographed. The presence of degenerating axons in 10 randomly selected fields, 50 × 80 μm, of each section (n = 6) of ON of each experimental group (n = 4), was examined and the number of axons with degenerating axon profiles, displaying myelin swelling and irregular debris, was counted in a masked manner and compared.

NGF-receptor Immunohistochemistry

Eyes were fixed overnight in 4% paraformaldehyde in 0.1 M PBS, pH 7.4, and then left overnight in 0.1 M PBS containing 20% sucrose. Coded 15-μm thick sections of each retina were cut with a cryostat at −20°C and immunostained with anti-TrkA (1:70; Upstate, Temecula, CA) and anti-p75 antibodies, previously produced.[27] For quantitative analyses, we counted labeled RGCs in 12 different areas (10.0 × 10^2/mm^2 each) of each retina, located approximately 300 μm from the centre of the optic disk, using a Zeiss microscope, equipped with a computerized image analysis system and a controlled motorized stage (IAS 2000, Delta Sistemi, Roma, Italy). The number of TrkA and p75 immunostained RGCs was defined as the percentage of immunostained cells compared with CV. All counts were performed in a masked manner.

Western Blotting

Retinal tissues were isolated and immediately sonicated. Tissue samples were homogenized in buffer (10 mM Tris-acetate, pH 7.4; 100 mM NaCl; 1 mM ethylenediamine-tetraacetic acid; 1 mM ethyleneglycol-tetraacetic acid; 1% Triton X-100; 10% glycerol; 0.1% sodium-dodecyl-phosphate, SDS; 20 mM $Na_4P_2O_7$; 2 mM sodium-orthovanadate, Na_3VO_4; 1 mM NaF; 2 μg/mL aprotinin; 1 mM phenylmethylsulfonyl fluoride; and 1 μg/mL leupeptin) at 4°C. After 12,000 rpm centrifugation for 20 minutes the supernatants were submitted to western blotting. Samples (30 μg of total protein) were dissolved in loading buffer (0.1 M Tris-HCl buffer, pH 6.8, containing 0.2 M dithiothreitol, 4% SDS, 20% glycerol, and 0.1% bromophenol blue), separated by 8% or 12% SDS-polyacrylamide gel electrophoresis, and electrophoretically transferred to polyvinylidine diflouride membrane overnight. The membranes were incubated for 1 hour at room temperature with blocking buffer constituted by 5% non-fat dry milk in TBS-T (10 mM Tris, pH 7.5, 100 mM NaCl, and 0.1% Tween-20). Membranes were washed 3 times for 10 minutes each at room temperature in TBS-T followed by incubation at 4°C with primary antibodies overnight (anti-TrkA and anti p75 antibodies purchased from SantaCruz, CA and anti-β-actin

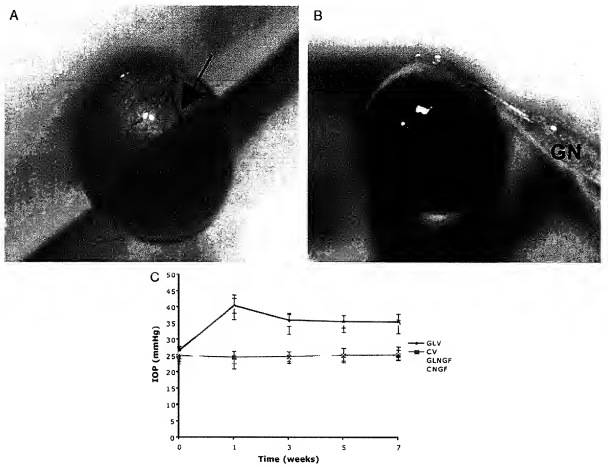


FIGURE 1. Representative microphotographs showing the episcleral vein (arrow) of rat eyes (A), the site of insertion of the glass microneedle (GN) in the episcleral vein (B), and the time-course IOP of rats injected and non-injected with hypertonic saline (C). As reported in this figure, the injection of hypertonic saline in the episcleral vein causes a significant elevation of IOP as compared with control, reaching peak after 1 week ($P<0.01$). As indicated, nerve growth factor (NGF) eye administration is unable to normalize the IOP. CNGF indicates control eye treated with NGF; CV, control eye treated with vehicle solution; GLNGF, glaucomatous eye treated with NGF; GLV, glaucomatous eye treated with vehicle solution; IOP, intraocular pressure.

from SIGMA, St Louis). Membranes were washed 3 times for 10 minutes each at room temperature in TBS-T and incubated for 1 hour with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG or HRP-conjugated anti-mouse IgG as the secondary antibody (Cell Signaling Technology, MA). The blots were developed with an enhanced chemiluminescent HRP substrate as the chromophore (Millipore, MA). The public Image J Software was used to evaluate band density, which was expressed as arbitrary units of grey level. The Image J program determines the optical density of the bands using a grey scale shareholding operation. The optical density of β-actin bands was used as a normalizing factor. For each gel per blot, the normalized values were then expressed as percentage of relative normalized controls and used for statistical evaluation. Statistical evaluations were performed using the StatView package for Windows. The data are expressed as means ± SEM. TrkA and p75 protein concentrations determined by western blot analyses were evaluated by computing analyses of variance. A P value of less than 0.05 was considered significant.

Analysis of Cell Death

For the determination of RGC death and survival, sections of the retina were immunostained with terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick-end labeling (TUNEL), a marker of apoptotic cell death.[30] Briefly, retinal sections were first incubated in a blocking solution (3% H_2O_2 in methanol) for 10 minutes at 15 to 25°C, incubated in a permeabilization solution containing PBS 1× with 0.1% Triton X-100 for 2 minutes at 4°C, and then labeled with an in-situ cell death detection kit (Roche Diagnostic, Boheringer, Mannheim, Germany) according to the manufacturer's instructions. DNA strand breaks were labeled and visualized with 0.4% diaminobenzidine-H_2O_2. TUNEL-positive cells with nuclear condensation or fragmentation were considered as apoptotic cells. The number of TUNEL-stained cells was expressed as RGCs per mm^2.

NGF Determination

Retinas from 24 animals (n = 6 retinas per each experimental group) were homogenized by ultrasonication

A

Concentration of NGF protein (pg/g) in the retina of glaucomatous and non-glaucomatous rats under different experimental conditions.

	Weeks after treatment			
Treatment	0	1	3	7
CV	87±8.5	86±9.2	89±7.5	94±8.3
CNGF	85±10	117±11.9	116±10.4	139±10.5
GLV	89±8.5*	98±7.5*	104±7.0*	62±5.2**
GLNGF	84±8.0[§]	112±9.9[§]	137±12.7 [§]	151±21.3***

CV=non-glaucomatous eye treated with vehicle; CNGF=non-glaucomatous eye treated with NGF; GLV= glaucomatous eye treated with vehicle; GLNGF=glaucomatous eye treated with NGF. Data are the average ± S.E.M. of 5 samples in each experimental group.
**p<0.01 compared to *; ***p<0.01 compared to [§].

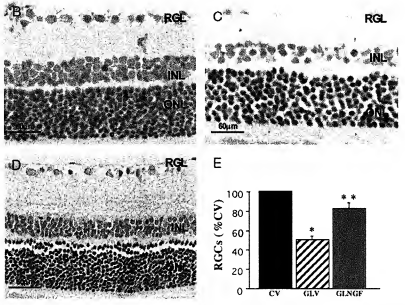


FIGURE 2. Time-course changes and statistic comparison between the end of treatment (7 wk) and 0 to 3 weeks reported in horizontal orientation. The concentration of NGF increases in the retina of glaucomatous retina (GLV) during the first 3 weeks (89±8.5, 98±7.3 vs. 104±7.0), thereafter the levels of NGF decreased significantly (62±5.2 vs. 104±7.0). In the glaucomatous retina treated with NGF (GLNGF), the level of NGF is elevated throughout the NGF treatment. An increase in NGF was also found in the retinas of normal rats treated with NGF (CNGF). Representative microscopic fields of normal (B), glaucomatous (C), and glaucomatous NGF-treated retinas (D), stained with hematoxylin-eosin showing the 3 retinal layers, the retinal ganglion layer (RGL), inner retinal layer (INL), and the outer retinal layer (ONL). Note that 7 weeks after hypertonic saline injection, the EIOP causes a marked decrease in the number of RGCs in the glaucomatous retina, compared with control retina. Quantitative evaluation (E) indicates that EIOP causes a statistically significant decrease in the number of RGCs compared with the control retina, whereas ocular administration of NGF largely prevented this decreases (*$P < 0.01$ compared to CV, and **$P < 0.05$ compared to GLV). B to D, Scale bars: 60 μm. EIOP indicates elevated intraocular pressure; NGF, nerve growth factor; RGCs, retinal ganglion cells.

in extraction buffer (10 mM Tris-acetate, pH 7.4; 100 mM NaCl; 1 mM ethylenediamine-tetraacetic acid; 1 mM ethyleneglycol-tetraacetic acid; 1% Triton X-100; 10% glycerol; 0.1% SDS; 20 mM $Na_4P_2O_7$; 2 mM sodium-orthovanadate, Na_3VO_4; 1 mM NaF; 2 μg/mL aprotinin; 1 mM phenylmethylsulfonyl fluoride; and 1 μg/mL leupeptin), centrifuged at 4°C for 20 minutes at 12,000 rpm. The concentrations of NGF in the supernatants were determined by the use of an enzyme-linked immunosorbent assay kit following the instructions provided by the manufacturer (NGF Emax Immunoassay System; Promega, Madison, WI).

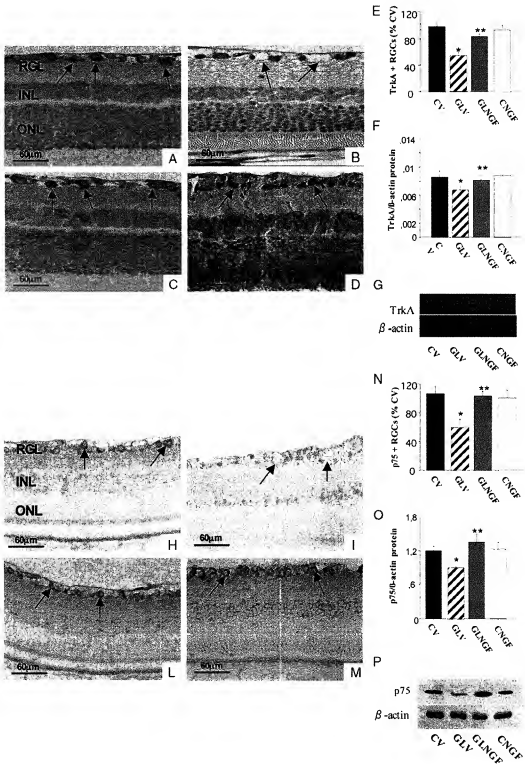
Statistical Analysis

All statistical evaluations were performed using the StatView package for Windows. The data are expressed as means ± SEM. A P value of less than 0.05 was considered significant. Post-hoc comparison within logical sets of means was performed using Tukey test.

RESULTS

EIOP After Hypertonic Saline Injection

Figure 1 shows the globes with the episcleral vein (arrow; Fig. 1A) and the site of injection (Fig. 2B) of hypertonic saline with a glass microneedle. As shown in Figure 1C, injection of hypertonic saline leads to elevation of IOP, reaching greatest increase 7 days postinjection (42.7 ± 3.7 mm Hg as compared with 26.9 ± 2.6 mm Hg of CV), and remained elevated for the following



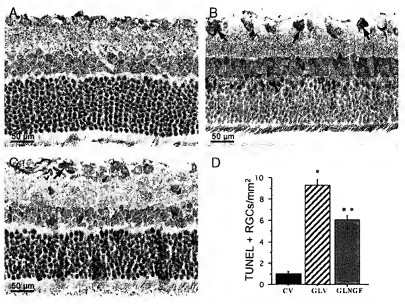


FIGURE 4. Representative retinal sections of control (A), glaucomatous (B), and glaucomatous eyes treated with NGF (C) stained by TUNEL, a marker for cell death. Note the presence of TUNEL-positive retinal ganglion cells (RGCs) (arrows) in glaucomatous eyes compared with control and glaucomatous eyes treated with NGF. TUNEL-positive cells in the retinal ganglion layer of glaucomatous eyes are indicated with arrows. These differences are statistically significant (D) [*P<0.01 vs. control eye treated with vehicle solution (CV); **P<0.05 vs. glaucomatous eye treated with vehicle solution (GLV)]. A to C, Scale bars: 50 µm. GLNGF indicates glaucomatous retina treated with NGF; NGF, nerve growth factor; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick-end labeling.

7 weeks. Ocular administration of NGF has no effect on IOP.

NGF and RGC Distribution in Glaucomatous Untreated and NGF-treated Eyes

Figure 2A reports the concentration of NGF in the retina with and without EIOP of NGF-treated and untreated rats. The level of NGF in the GLV retina decreased compared with baseline levels, at 7 weeks after induction of IOP (from 89 ± 8.5 to 62 ± 5.2 pg/g; $P < 0.05$). Ocular administration of NGF to glaucomatous eye (GLNGF) for 7 weeks enhanced retinal NGF levels (from 84 ± 8.0 to 151 ± 21.3 pg/g; $P < 0.01$). An increase in retinal NGF was also found in the control retina treated with NGF (CNGF; from 85 ± 10 to 139 ± 10.5 pg/g; $P < 0.05$).

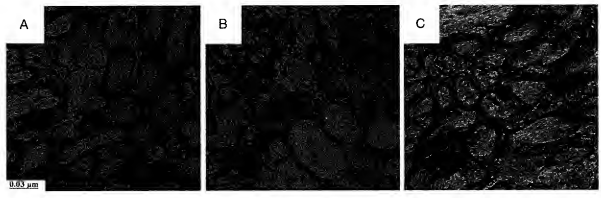
Histologic analysis of the retina shows cell loss in the retinal ganglion layer after hypertonic saline injection: GLV (Fig. 2C) compared with CV (Fig. 2B). Ocular administration of NGF for 7 consecutive weeks prevented RGC loss in GLNGF (Fig. 2D). Quantitative analyses indicate that EIOP causes nearly 50% loss of the RGCs and that after NGF treatment, this loss is less than 20% (Fig. 2E).

The EIOP Causes Loss of NGF Receptors in RGCs

Under normal conditions, RGCs of rats express TrkA and p75 NGF receptors (Figs. 3A, H). Seven weeks after induction of EIOP, the number of TrkA-positive RGCs

◀

FIGURE 3. Expression of TrkA and p75 receptors in retina of rats with EIOP treated and untreated with NGF. Representative microscopic fields of TrkA-immunostained control (A), glaucomatous (B), glaucomatous NGF-treated (C), and non-glaucomatous NGF-treated (D) in the RGL. These immunostained preparations indicate a reduced presence of TrkA-positive RGCs (arrows) in rats with EIOP (B), compared with controls (A) and that ocular NGF administration nearly prevented this reduction (C). Quantitative analysis (E) revealed that the decrease of TrkA-positive cells in glaucomatous retina and the effect on NGF are statistically significant (*$P < 0.01$ vs. CV; **$P < 0.05$ vs. GLV). Western blotting analysis of TrkA protein expressed in the glaucomatous retina treated and untreated with NGF, compared with their controls (F and G). The result indicates that TrkA protein decreases in glaucomatous retina and NGF administration in glaucomatous retina reduces this deficit (*$P < 0.01$ vs. CV; **$P < 0.05$ vs. GLV). Representative microscopic fields of p75-immunostained control (H), glaucomatous (I), glaucomatous NGF-treated (L), and non-glaucomatous NGF-treated (M) in the RGL. These immunostained preparations revealed a decreased expression of p75-positive RGCs (arrows) in rats with EIOP (I), compared with controls (H) and that ocular NGF administration nearly prevented this reduction (L). Quantitative analysis (N) revealed that the decrease of p75-positive cells in glaucomatous retina and the effect on NGF are statistically significant (*$P < 0.01$ vs. CV; **$P < 0.05$ vs. GLV). Western blotting analysis of p75 protein expressed in the glaucomatous retina treated and untreated with NGF, compared with their controls (O and P). The data obtained indicates that p75 protein decreases in glaucomatous retina and NGF administration in glaucomatous retina reduces this effect (*$P < 0.01$ vs. CV; **$P < 0.05$ vs. GLV). A to D and H to M scale bars: 0.03 µm. CNGF indicates control eye treated with NGF; CV, control eye treated with vehicle solution; EIOP, elevated intraocular pressure; GLNGF, glaucomatous retina treated with NGF; GLV, glaucomatous eye treated with vehicle solution; INL, inner retinal layer; NGF, nerve growth factor; ONL, outer retinal layer; RGCs, retinal ganglion cells; RGL, retinal ganglion layer.



D

Axons of the optic nerve of control (CV), glaucomatous treated with vehicle (GLV) and glaucomatous treated with NGF (GLNGF) rats showing degenerative profiles, as revealed by ultrastructural analysis

type of treatment	ON section/rat	Area/section/µm2	degenerating profile/area
CV	6	40000	15.0±7.3
GLV	6	40000	230.0±15.4**
GLNGF	6	40000	60.0±5.4*

**p<0.01 compared to CV and *p<0.05 to CV

FIGURE 5. Representative electron-microscopic sections of control (A), glaucomatous untreated (B), and glaucomatous NGF-treated ON (C), 7 weeks after the induction of elevated intraocular pressure. Note retinal ganglion cell axons in the ON of glaucomatous eye (B) showing varicosities, swellings, and vacuolization (arrows), compared with control ON (A). In the glaucomatous ON treated with NGF, these degenerating signs are greatly reduced or nearly absent (C). These differences are statistically significant (D) (*$P < 0.01$ vs. CV; **$P < 0.01$ vs. GLV). A to C scale bars: 0.03 µm. NGF indicates nerve growth factor; ON, optic nerve.

decreases (Fig. 3B vs. Fig. 3A) and this decrease is prevented by ocular administration of NGF (Fig. 3C vs. Fig. 3B). As reported in Figure 3E, these differences are statistically significant (GLV vs. CV: *$P < 0.01$; GLNGF vs. GLV: **$P < 0.05$). Moreover, results of western blotting analysis, shown in Figures 3F and G, indicated that the TrkA protein expressed in the glaucomatous retina decreases and that NGF administration nearly prevented this decrease.

Figure 3 reports also the effect of EIOP on the expression of p75 receptor by RGCs. EIOP reduces the number of p75-positive cells (Fig. 3I vs. Fig. 3H) and this decrease is prevented by ocular NGF administration (Fig. 3L vs. Fig. 3I). As reported in Figure 3N, both the decrease of p75 expression in glaucomatous retina and the protective effect of NGF administration are statistically significant (GLV vs. CV: *$P < 0.01$; GLNGF vs. GLV: **$P < 0.05$). Western blotting analysis, shown in Figures 3O and P, showed that the p75 protein present in the retina decreases in glaucomatous retina and that this decrease is nearly prevented by ocular NGF administration.

NGF Ocular Administration Prevents Apoptotic RGC Death

To assess whether the reduced RGCs number was due to cell death, we next investigated the expression of biomarkers involved in cell death and cell survival. The RGCs of rats with EIOP are markedly stained by TUNEL (arrows in Fig. 4B), a biomarker of cell death, and are absent in control retina (Fig. 4A) and nearly absent in NGF-treated retinas (Fig. 4C). Quantitative analysis reported in Figure 4D revealed that the effect of NGF in preventing RGC apoptotic death is statistically significant ($P < 0.01$).

NGF Administration Reduces the Number of Degenerating ON Axons Induced by EIOP

Figure 5 shows representative electron microscopic sections of normal (Fig. 5A), glaucomatous (Fig. 5B), and NGF-treated glaucomatous (Fig. 5C) ON of a rat 7 weeks after induction of EIOP. As indicated by arrows, the ON of glaucomatous NGF-untreated rat shows degenerating axon profiles, characterized by varicosities or swelling, vacuolization, debris, and also signs of degenerating cells, most probably oligodendrocytes, nearly absent in the control and NGF treated axons. Quantitative determination reported in Figure 5D indicates that these differences are statistically significant ($P < 0.01$).

DISCUSSION

Using a rat model of GL induced by EIOP, we have investigated the role of NGF on damaged RGCs and ON axons. The results show that EIOP causes a transient local increase of NGF in the retina, followed by a significant decreases leading ultimately to RGC death through apoptotic mechanisms. We found that EIOP causes loss of RGCs, degeneration of ON axons, and a significant reduction of NGF, associated with a marked lower expression

of low and high NGF-receptor by RGCs. EIOP causes a significant increase of TUNEL staining by RGCs, suggesting that degeneration of RGCs occurs through apoptotic mechanisms. These changes are markedly reduced by daily administration of eye NGF application, suggesting that NGF can protect the progressive degeneration of RGCs induced by EIOP.

Moreover, this study revealed that NGF delivered as eye drops protects RGCs and ON axons from degeneration and this noninvasive delivering method exerts its protective action in the absence of side effects. These observations support and extent earlier findings that NGF and NGF receptors play a critical protective action on RGCs.[15–17,20,21] More specifically, they indicate that shortage of NGF combined with abnormal expression of NGF-receptors are important key events that can lead to progressive RGC degeneration in GL and that NGF ocular administration can reduce or prevents these deleterious effects.

The critical role of NGF and its receptors in cell survival and cell death are supported by a number of other studies demonstrating that the biologic effect of NGF is mediated by competition between the low-affinity p75 receptor and the high-affinity TrkA receptor[31] and the number of NGF-receptive RGCs can be critically dependent on such feedback loop. This dynamic mechanism regarding the role of NGF and/or NGF-receptor interaction can occur also in diabetic NGF-responsive cells. For example, recent studies showed that NGF supplementation produces significant changes in NGF-receptor expression, leading to a 19-fold increase in TrkA/p75.[32,33]

The role of NGF on retinal cells has been suggested by a number of other earlier and recent findings. Thus, intraocular administration of NGF has been shown to reduce rat RGC degeneration after ON lesion,[14] in rabbit ocular hypertension,[19] and in rats with inherited retinopathy.[17] It has been reported that administration of high molecular weight protein to the ocular surface can reach the retina and brain neurons through[34,35] and that topical ocular administration of NGF can be a noninvasive approach to deliver NGF to the posterior portion of the eye and into the brain. These latter studies have demonstrated the protective role of eye NGF application on retinal cells not only in laboratory animals,[36–38] but also in humans.[39] Indeed, in a recent observation in 3 patients with progressive visual-field defects and severe abnormalities in photopic electroretinogram and visual evoked potential responses and dysfunction of the innermost retinal layers, ocular NGF application improved mean visual field defects in 2 patients, and stabilized the defect in the third patient. Improvement of visual field persisted 90 days after discontinuation of treatment, indicating that changes induced by NGF had a long-lasting effect.[39]

Recently, studies by Rudzinski et al[40] and Shi et al[41] performed in animal model of ocular hypertension or GL lead to the hypothesis that neither NGF nor antagonist of the proapoptotic p75 protect damaged RGC. Our results are somehow in contrast with their findings. Why our findings are different is not clear. We believe, based also on the available data that these differences can be because of: (a) the different animal model of GL we used; (b) the time and dose of NGF treatment; and (c) a more detailed structural and biochemical analysis or a combination of (a), (b), and (c). Moreover, it should be also taken into consideration that NGF has been shown to protect damaged RGCs after ON section,[14] after ocular ischemia,[16] ocular hypertension,[40] and inherited retinitis pigmentosa.[17]

A key question raised by our observations is the mechanism through which NGF administration protects RGCs without lowering the IOP. The evidence that patients with low IOP still experience visual loss, and others with GL and ON degeneration display normal IOP,[4,5] suggests that the EIOP might not be the only mechanism through which RGCs degenerate. Thus, changes in the neuronal environment, such as a neurotransmitter imbalance, influx of calcium into the cells, formation of free radicals, and depletion of growth factors can take part in retinal degeneration and RGC damages induced by elevated IOP. This hypothesis is suggested by observation after systemic application of brimonidine,[42] chondroitin sulphate proteoglycan,[43] or memantine, an *N* methyl-D-aspartate open channel blocker.[44] On the basis of the available data that RGCs are highly receptive to NGF, a reasonable hypothesis is that the ocular NGF application reaches the posterior segment of the eye bindings to retinal cells and renders more resistant RGCs to the damage induced by EIOP. Moreover, the possibility that NGF can exert its action through other growth factors, including brain-derived neurotrophic factor, cannot be excluded.[18,25]

In summary, our data suggest that the reduced presence of NGF and the loss of NGF receptors in retinal cells is an important step in the progression of GL. The reduction of degenerative events in RGCs and ON after ocular NGF application suggests that NGF represents a possible useful molecule for GL by protecting RGC death and ON axon loss.

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Annex 1

USE OF NERVE GROWTH FACTOR FOR THERAPY OF INTRAOCULAR TISSUE PATHOLOGIES

Experimental Report on the Passage of NGF through the Ocular Tissues

We used two techniques to evaluate the passage to the retina and optic nerve of different concentrations of NGF eye drops administered in the conjunctival fornix.

First we used autoradiography by means of ^{125}I-NGF incorporation. ^{125}I-NGF was radioiodinated with Na ^{125}I (Amersham) by the chloramine-T procedure and purifed by Sephadex G-25 column chromatography. The specific activity was 1.0-1.5 Ci/mmol. 10 µl of ^{125} I-NGF eye drop at four different concentrations (1 µg/ml, 10 µg/ml, 200 µg/ml, and 500 µg/ml) was administered in the conjunctival fornix of twelve rabbits (3 animals for each concentration). The latter were and sacrificed 2, 6 and 24 hours after the NGF administration. The eyes were removed and fixed in 4% paraformaldehyde in phosphate buffer 100mM, pH 7.4. The eyes subsequently were placed in a 30% buffered sucrose solution for 24hrs. Sections of eyes (10 µm thickness) were cut and used for autoradiography. Briefly, slides were coated with nuclear tracking emulsion Ilford K2 (Ilford), developed using Kodak D19 developer after 1 month exposure and counterstained with Toluidine blue.

Our data show that labeled NGF was observed in the optic nerve 2 hours afer the conjunctival administration when administered at a concentration of 10 µg/ml, 200 µg/ml and 500 µg/ml. Labeled NGF increased 6 hours after the administration and was no more present 24 hours later. In the retina labeled NGF was observed only 6 hours after conjunctival administration at the same

concentration observed for optic nerve (10 μg/ml, 200 μg/ml and 500 μg/ml).

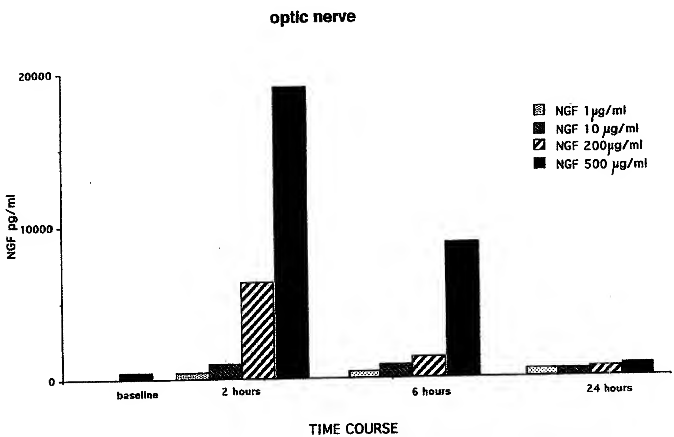
No evidence of a passage of labeled NGF in the retina or optic nerve was observed when the eye drop contained the lower concentration of 1 μg/ml of NGF.

This data was confirmed by ELISA technique. NGF eye drop at four different concentrations (1 μg/ml, 10 μg/ml, 200 μg/ml, and 500 μg/ml) was administered in the conjunctival fornix of sixty rabbits and the animals were sacrificed 2, 6 and 24 hours after the administration.

Our results show that NGF administration in the conjunctival fornix reached the retina and the optic nerve 2 hours after the administration and returned to the baseline values at 24 hours (see figures 1 and 2). The levels of NGF were increased in the retina and optic nerve depending on the concentration of the eye drop.

No effect was observed for the lower concentration (1 μg/ml) at any time point in both retina and optic nerve. This last observation is in line with the well known activity of NGF. In fact the active concentration of NGF for *in vitro* culture is around 250 ng/ml while the NGF is used at a concentration of approx. 0.1-1 μg/ml in animal study using local injection (intracerebral, intraocular injection). If it is considered the eye drop administration of NGF to reach the posterior segment it is likely to hypothesize a dramatic decrease of the NGF reaching the retina and optic nerve, as shown by our study.

Therefore the concentration of 0.02 and 0.04 μg/ml, used by Okamoto in his *in vivo* study, appears to be completely unable to induce any detectable effects, in line with our data showing no activity of eye drop with a concentration at least 25 times higher (1 μg/ml).



optic nerve
20000
10000
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NGF pg/ml
NGF 1μg/ml
NGF 10 μg/ml
NGF 200μg/ml
NGF 500 μg/ml
baseline
2 hours
6 hours
24 hours
TIME COURSE

retina
NGF pg/ml
20000
10000
0
baseline
2 hours
6 hours
24 hours
TIME COURSE
NGF 1µg/ml
NGF 10 µg/ml
NGF 200µg/ml
NGF 500 µg/ml

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LOOKING AHEAD

NERVE GROWTH FACTOR EYE DROPS TO TREAT GLAUCOMA

NGF eye drops have the ability of targeting the optic nerve and brain, thus opening-up avenues of research in pursuit of a novel drug for neurodegenerative diseases.

by Alessandro Lambiase, Flavia Mantelli and Stefano Bonini

Nerve growth factor (NGF), the first and most well-known member of the family of neurotrophins (NTs), is a pleiotropic factor that extends its biological activity from the central and peripheral nervous systems to the immune, endocrine and visual systems (1). Signals mediated by NGF are propagated by two distinct receptors: Trk-A (a tyrosine kinase receptor) and p75 (a member of the tumor necrosis factor receptor superfamily) (2, 3). The duration and magnitude of NGF signaling depends on the ratio of Trk-A and p75 codistributed on the cell surface (4). Functionally, signaling by Trk-A and p75 may be synergistic, independent or antagonistic. Interestingly, these opposing NGF effects, ranging from cell growth, differentiation, survival and cell death, are apparently not shared by the other NTs, although all bind p75. From the discovery of NGF in the 1950s, increasing evidence has demonstrated that NGF is able to aid survival of neurons in vitro and during neurodegenerative diseases in experimental animal models. Preliminary data in a limited number of patients also support these experimental results (5). Thus, NGF has raised expectations that its clinical application to neurodegenerative diseases, such as Alzheimer's and Parkinson's disease, might provide an effective therapy for these conditions still considered untreatable (6).

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During the 1980s several researchers highlighted the role of NGF in the development and pathophysiology of the retina and optic nerve, showing the therapeutic effectiveness of intraocular NGF delivery in experimental models of several ocular diseases, such as ischemic retinopathy and optic nerve trauma. These studies demonstrated that this protective role of NGF was primarily exerted on the retinal ganglion cell (RGC) layer. Based on this evidence, our group tested NGF on an animal model of ocular hypertension, confirming the previous reports on the beneficial effects of NGF on RGC and widening its possible applications from acute optic nerve damage to chronic optic nerve diseases, such as glaucoma (7, 8). At this point, NGF was becoming a promising new drug for a wide spectrum of ocular diseases. However, researchers soon faced the first difficulties related to drug delivery routes in chronic disease. Only more recently, pharmacodynamic studies documented that NGF administered on the ocular surface as eye drops is able to reach the posterior ocular segment where it inhibits RGC degeneration in animal models of glaucoma, opening new perspectives for the treatment of optic nerve, retina and brain neurodegenerative diseases (7, 9, 10).

NERVE GROWTH FACTOR AND THE POSTERIOR OCULAR SEGMENT

The eye is a complex organ composed of a considerable number of structures, which can be grossly divided into the components of an anterior ocular segment and a posterior ocular segment. The posterior ocular segment comprises the vitreous body, the retina, the choroid and the optic nerve that carries the visual stimuli from the eye to the central visual pathway (Fig. 1).

The first evidence of NGF's involvement in the visual system has been demonstrated on the retina and the central visual pathway.

SUMMARY

Since the discovery of nerve growth factor (NGF) in the 1950s, scientists have had great expectations for NGF in the treatment of neurodegenerative diseases. The main obstacle to overcome was the blood-brain barrier, which NGF does not cross when administered systemically. Our research group has recently demonstrated that topically instilled NGF eye drops are effective in treating several ocular diseases such as neurotrophic and autoimmune corneal ulcers. Pharmacodynamic studies have also shown that NGF eye drops have the ability to target the optic nerve and brain, thus finally opening-up avenues of research in pursuit of a novel drug for neurodegenerative diseases. In this review, clinical trials of systemically and topically administered NGF are discussed, as well as recent progress in the production of biologically active recombinant human NGF and innovative delivery routes. The pharmacological activity of NGF eye drops in ocular surface, retina and optic nerve diseases are also discussed.

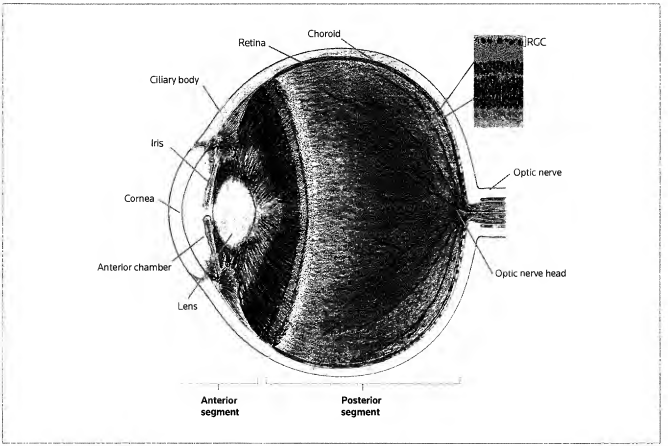

Figure 1. Anatomy of the eye and visual pathway. A side view of the human eye is shown. The eye is a complex organ composed of an anterior and a posterior ocular segment. The cavity of the posterior ocular segment is occupied by the vitreous body, a clear gelatinous substance facing the retina. A histological section of the retina, stained with hematoxylin-eosin, is shown in the insert. The optic nerve head receives the retinal ganglion cell (RGC) axons, which form the optic nerve and carry the visual stimuli from the eye to the central visual pathway.

In the 1970s, it was shown that NGF and NGF receptors (NGFR) are widely expressed in the central visual pathway (lateral geniculate nucleus and visual cortex), as well as in the optic nerve and retina (1). Recently, it has also been found that NGF and NGFR are expressed by all the intraocular tissues, including lens, vitreous, choroid, iris and trabecular meshwork (11).

NGF has been found to strongly affect visual pathway development and plasticity. Specifically, NGF acts as a differentiation factor in the development of the visual cortex (12). In fact, during visual system development, NGF, Trk-A and p75 are localized in numerous visual centers, from the visual cortex to the retina, where NGF modulates neuritis outgrowth, survival and selective apoptosis (12-14). In rats undergoing experimental monocular sensorial deprivation (occlusion of one eye), NGF treatment restored visual acuity and visual cortex neuron distribution, maintaining the ocular dominance and neural functions in the lateral geniculate nucleus, as compared to the control eye (12, 15).

NGF modulates retina and optic nerve development/differentiation and promotes the survival and recovery of RGCs. The first evidence for an NGF/NGFR pathway expression in the retina came from observations in chicken and developing rat retina (16, 17). In the retina, NGF is produced and utilized by RGCs, bipolar neurons as well as glial cells, in a local paracrine/autocrine fashion. A retrograde/anterograde transport along the axons of the RGCs (optic nerve) and geniculate nucleus has also been reported (14). After NGF administration at the site of lesion, retrograde/anterograde transport was also demonstrated in the optic nerve of goldfish and rats (14, 18). Subsequently, NGF production from RGCs, Müller cells and pigmented epithelium of adult rats was also reported, as well as Trk-A NGFR expression in the pigmented epithelium and Müller cells of rat adult retina (14, 19). NGF also affects retinal plasticity, with massive cell death observed during retinal development associated with a consistent p75 expression. Conversely, several experimental studies demonstrate that the NGF/Trk-A pathway promotes RGC survival. In fact, intraocular injection of NGF promoted recovery of damaged RGCs after ischemic injury, optic nerve transection and ocular hypertension, in contrast to the exacerbating effects of the administration of neutralizing anti-NGF antibodies (8, 20, 21). Recently, it has been demonstrated that

intravitreal injection of exogenous NGF protects retinal cell degeneration and apoptosis in experimental retinal detachment (22). In addition to the original discovery of NGF as a survival and neurite growth-promoting factor, in the retina NGF appears to exert multiple effects on different cell types, including proliferation, transmitter synthesis, cytoskeletal changes, synaptic transmission, reorganization and plasticity (1). NGF expression was found to be decreased in the retina of mice affected by an inherited retinal degeneration that resembles retinitis pigmentosa (23). Exogenous retrobulbar or intraocular NGF administration significantly delayed retinal degeneration in these mice (24). In diabetic rats, intraocular administration of NGF triggered recovery of damaged retina (25). Moreover, in this animal model both RGCs and Müller cells underwent apoptosis by overexpression of p75, while exogenous NGF treatment appeared to prevent the loss of RGC, Müller cells and vascular pericytes (25). In addition, the presence of NGF receptors in the trabecular meshwork and evidence suggesting a role of NGF in regulating genes involved in glaucoma pathogenesis, cannot exclude other mechanisms of action of NGF in this disease (26).

GLAUCOMA AS A POTENTIAL TARGET OF NGF TREATMENT

Glaucoma is the leading cause of irreversible blindness in the world (27). This chronic and progressive disease is an optic neuropathy characterized by degeneration of the RGC and loss of axons of the optic nerve with a progressive and consequent deficit of the peripheral and central visual field (28). Early diagnosis of an elevated intraocular pressure, the only risk factor that can be actually modified, may significantly reduce the risk of progressive nerve degeneration, but to date there is no treatment available to restore neural function (29). In glaucoma, up to 20% of patients show progression of visual field defects with RGC and optic nerve degeneration despite successful management of ocular hypertension (30). In fact, elevated intraocular pressure is thought to be only the *primum movens* that triggers a cascade of events leading to optic nerve damage (31).

Many similarities between glaucoma and Alzheimer's disease go far beyond the challenges encountered in their treatment: 1) RGCs die by apoptosis in glaucoma through activation of specific caspases, which are also activated in Alzheimer's disease; 2) caspase activation with cleavage of amyloid precursor protein has been shown to upregulate β-amyloid production in Alzheimer's disease and in animal models of glaucoma; 3) age-related mitochondrial dysfunction plays a key role in the etiology of both neurodegenerative disorders; 4) elevated glutamate and nitric oxide synthase upregulation with reactive oxygen species formation have been implicated in both glaucoma and Alzheimer's neurotoxicity; and 5) glutamate toxicity is involved in both glaucoma and Alzheimer's synaptic dysfunction (32, 33). All of these similarities have led glaucoma to be dubbed the "ocular Alzheimer's disease" (32).

An approach that would vastly improve the treatment of this challenging disease would involve neuroprotection with exogenous neurotrophic factors. In fact, neuroprotection has gained substantial interest in recent years as a therapeutic approach to prevent neuronal degeneration and loss of function in glaucoma (31). Neuroprotective therapies currently under investigation to restore retinal/neural function include memantine, NTs, erythropoietin, reactive oxygen species' scavengers and even vaccine therapies (34, 35). Nevertheless, results of randomized clinical trials conducted so far did not meet the expectations.

Intracerebral administration of NGF has been shown to be beneficial in Parkinson's and Alzheimer's patients, and intraocular administration of NGF in animal models has been shown to inhibit RGC degeneration after mechanical, ischemic or hypertensive injury (8, 20, 21, 36). Recently, it has been demonstrated that murine NGF administered topically to the eye rescued RGCs from apoptosis in a rat model of glaucoma (7). In this experimental model of glaucoma, a single injection of hypertonic saline into the episcleral veins of rat eyes induced chronic elevation of intraocular pressure, optic nerve degeneration and selective RGC loss by apoptosis, the sum effects of which resemble human glaucoma (7, 37). The beneficial effect of NGF eye drops on RGC survival was demonstrated to be due to inhibition of apoptosis, as shown by the reduction in TUNEL RGC immunostaining and the greater retinal Bcl-2/Bax ratio. It is known that RGCs express NGF receptor (Trk-A) and that NGF binding to Trk-A upregulates Bcl-2 protein, which protects cells from apoptosis by preventing caspase activation. The crucial role of Trk-A/p75 ratio expression in determining the fate of RGC is supported by the evidence that in animal models of glaucoma, an imbalance of Trk-A/p75 ratio is associated with progression of retinal damage. Trk-A and p75 do not compete to mediate NGF signaling, but physically interact forming heterodimers (depending on their ratio) to determine NGF's biological effect (38). The effect of exogenous NGF administration in inducing an increase in Trk-A may result in changes of Trk-A/p75 interaction, justifying the protective effect on RGC and avoiding the potential proapoptotic effects of p75 binding. In line with this hypothesis, Lebrun-Julien et al. recently demonstrated that inhibition of p75 increases the effects of NGF and Trk-A agonists in promoting RGC survival (39). Another study on an animal model of glaucoma provides discrepant results that may be due to different study settings, highlighting the importance of the biological characteristics of NGF and its routes of administration (40). An increase of intraocular NGF during the progression of retinal degeneration was also observed during experimental glaucoma (21). These observations are in line with previous reports showing that intraocular injection of NGF promoted recovery of damaged RGCs after ischemic injury, optic nerve transection and ocular hypertension, in contrast to the detrimental effects of the administration of neutralizing anti-NGF antibodies (21).

Recently, it has been shown that NGF eye drop treatment induced long-lasting improvements in visual field, optic nerve function as measured by elecrofunctional parameters, contrast sensitivity and visual acuity in three patients with advanced glaucoma. These pivotal clinical data are in line with the known NGF effects on protection against neural apoptosis, and also on promotion of neural plasticity and axonal regeneration at the central and peripheral nervous systems (41). In fact, NGF acts on numerous levels to promote neuronal recovery following ischemic and chemical injuries: through a neosynaptogenetic mechanism, by directly affecting precursor cells and/or by induction of other growth factors, including brain-derived neurotrophic factor (BDNF) as demonstrated in the CNS and retina (42-45). In addition, the improvement of psycho-functional visual tests following

NGF eye drop treatment may be related to observations that 1) NGF topically applied on the ocular surface reaches retina, optic nerve and also brain area; 2) NGF is involved in the pathological changes observed in the superior geniculatus nucleus during experimental glaucoma; and 3) NGF affects visual cortical neuronal activity reflected by receptive field size, orientation selectivity, visual acuity, response latency and habituation (9, 10, 46, 47).

NGF EYE DROP USE IN PATIENTS

Topical NGF application has been tested in patients affected by a number of pathologies of the anterior segment by performing open clinical trials on small patient populations. Among all the potential therapeutic actions of NGF, the effectiveness in restoring corneal sensitivity makes this factor the only medical treatment currently available for neurotrophic keratitis, a disease caused by an impairment of corneal sensory nerves. The cornea, being an avascular structure, is unique in its dependence on sensory nerves for maintenance of trophism and for healing (48). Nerve damage leads to the development of a corneal lesion with markedly decreased healing properties (neurotrophic keratitis) (49). In 1998 the first open trial using murine NGF eye drops for the treatment of 12 patients affected by neurotrophic corneal ulcers unresponsive to standard therapy was published by our group (50). The study demonstrated that 2 to 6 weeks of NGF treatment induced corneal healing in all the patients associated with an improvement of corneal trophism, sensitivity and visual acuity. No relevant systemic or local side effects were reported in these patients. In line with this safety profile of NGF eye drop treatment, no side effects were reported in healthy corneas of glaucoma patients treated for 3 months (7). Two years later the same authors confirmed the therapeutic action of NGF eye drops on a larger population of 46 patients affected by stage 2 and 3 neurotrophic keratitis (51). During a follow-up period of 2 years, no side effects were observed and only 15% of the patients showed a relapse of the corneal lesion that healed following a new NGF treatment period. Recently, Tan et al. confirmed the effectiveness of NGF eye drop treatment in a child affected by a congenital form of neurotrophic keratitis unresponsive to any treatment (52). Treatment with murine NGF eye drops did not generate the development of autoantibodies against NGF in any patient (50-52).

More recently, the corneal healing effect of NGF eye drops has been tested in patients undergoing anterior segment surgery (including cataract surgery) and in patients affected by autoimmune corneal ulcers and by vasculitic and pressure skin ulcers (53, 54). Once again, NGF eye drops proved to be safe and efficient, and were found to play a role in the modulation of the immune and inflammatory reaction of the ocular surface (55).

Taken together, these results demonstrate that NGF eye drops can be considered a safe therapy without side effects that could limit their application in chronic diseases such as Alzheimer's disease and glaucoma.

PHARMACOLOGICAL DEVELOPMENT OF NGF

Clinical applications of NGF have so far been hindered by the difficulty to produce large amounts of biologically active recombinant human NGF (rhNGF) and in the case of diseases of the CNS, by its low permeability through the blood–brain barrier (56). Most of the clinical evidence of NGF efficacy has been obtained with murine-derived NGF, which cannot be routinely used for human therapy in spite of its documented safety (5, 7, 50, 51). For therapeutic purposes, large amounts of rhNGF are required. Molecular cloning and recombinant DNA technologies allow the production of a wide range of pharmaceutical products currently used for human healthcare (e.g., human insulin, interferons and vaccines). These technologies have been applied, so far unsuccessfully, also for production of rhNGF and no rhNGF is currently available for therapeutic purposes. The technology of rhNGF preparation is quite complex. In fact, β-NGF is a 26 kDa homodimer protein of 118 amino acids that is cleaved from a 241 amino acid precursor molecule, including a signal-sequence and a propeptide. The biological activity of mature NGF relies on formation of three disulphide bonds and a cysteine-knot within two chains, after cleavage of the signal- and propeptide sequences (57). This process requires correct post-translational modifications, such as *N*-glycosylation of pro-NGF, essential for efficient exit from the endoplasmic reticulum to the Golgi apparatus and its subsequent processing and secretion (58). So far, several attempts have been made to produce rhNGF in different cellular systems including *Saccharomyces cerevisiae* and *Escherichia coli* inclusion bodies, as well as insect and mammalian cells. Subsequently, in the 1990s the first clinical trials using rhNGF started: Two phase II clinical trials demonstrated that systemic administration of rhNGF was effective at improving both diabetic- and HIV-related neuropathy, having a painful sensation at the site of NGF injection as the only side effect (59, 60). However, a large-scale phase III clinical trial of 1,019 patients randomized to receive either rhNGF or placebo for 48 weeks failed to confirm the previous indications of efficacy. Among the explanations offered for the discrepancy between the two sets of trials were a robust placebo effect, inadequate dosage, different study populations and changes to the formulation of rhNGF for the phase III trial (61). Another reason could be due to the documented problems of low activity or low yields of rhNGF production. In fact, most of the rhNGFs commercially available for research purposes possess in vitro biological activity (differentiation of PC12 cells), but low activity in vivo compared to murine NGF. Evidence indicates that mammalian cells are the only appropriate system for production of biologically active rhNGF, as they can correctly process (fold, glycosylate and cleave) the precursor and secrete the mature protein. However, the yields of production are low and insufficient for therapeutic purposes. Recently, patented rhNGF production technologies represent promising tools to begin a new era of clinical trials in NGF research (62).

Once the goal of active rhNGF production is achieved, the next major limitation to the use of NGF as a pharmaceutical treatment for brain and ocular neurodegenerative diseases, as well as for retina and optic nerve pathologies, will be related to the blood–brain and blood–ocular barriers that inhibit NGF passage when systemically administered. Several attempts have been made to obtain biologically active levels of NGF in the brain: 1) intracerebral or intravitreal NGF infusion, invasive procedures that cannot be considered a gold standard treatment; 2) slow-release biodegradable implants, a technique that may be useful only when a local source of NGF is required for a limited

period of time; 3) carrier-mediated transport across the blood–brain barrier, a promising method based on coupling NGF to a carrier that enables transfer across this barrier (such as an antibody to the transferrin receptor); 4) ex vivo NGF gene delivery/grafting NGF-producing cells, a technique already used with encouraging results in a phase I clinical trial on 8 individuals with mild Alzheimer's disease; 5) direct gene transfer to the brain (or retina) to achieve a long-lasting local increase in NGF levels, and to restrict protein expression to specific cell types to avoid any potential side effects; 6) developing low-molecular-weight NGFR agonists capable of passing the blood–brain barrier; 7) controlling NGF endogenous production with pharmacological treatments capable of enhancing endogenous NGF synthesis, storage and release; 8) alternative delivery routes such as topical NGF administration. Recently, it has been demonstrated that both nasal and ocular topical NGF administration allow NGF to reach brain areas, inducing an increase of choline acetyltransferase activity in cholinergic nuclei and counteracting neural degeneration in animal models of Alzheimer's disease (36, 56, 63-68). Indeed, a pharmacokinetics study has shown that NGF eye drops administered on the ocular surface induces an increase of NGF levels in both retina and optic nerve (9). This increase of NGF in the retina may derive from 1) a direct passage through the conjunctiva and sclera; 2) NGF passage through the retrobulbar space and its retrotransport by the optic nerve to RGCs; 3) systemic absorption and subsequent NGF passage through the blood–ocular barrier. NGF administered as eye drops is biologically active, inducing an increase of Trk-A and BDNF synthesis in the retina (7). These data thereby suggest a novel, simple, noninvasive and effective delivery route of NGF for a wide spectrum of injuries and diseases in the CNS and in the ocular posterior segment.

CONCLUSIONS

Once regarded solely as a target-derived survival factor for the nervous system, NGF is now known to exert different roles in modulating the homeostasis of the ocular surface, as well as the retina and optic nerve. In fact, NGF affects corneal and conjunctival epithelium, fibroblasts and endothelium, immune cells, sensory nerves, tear production, but also RGCs, photoreceptors and optic nerve. As a consequence, NGF is involved in several human diseases with different etiopathologies, from the anterior and posterior ocular segment (Table I), to autoimmune and degenerative diseases. Most of the experimental evidence shows an efficacy of NGF treatment in degenerative diseases of the retina, such as glaucoma and retinitis pigmentosa, as well as of the cornea, such as neurotrophic keratitis.

Preliminary clinical trials, performed in severe ocular diseases unresponsive to any standard treatment, confirm the therapeutic efficacy and safety of NGF eye drop treatment. The results from these first clinical trials suggest that further multicenter, double-blind, randomized trials should be carried out. The evidence that NGF administered on the ocular surface reaches retina and optic nerve, as well as novel experimental and preliminary clinical data, suggest a potential use of NGF eye drops for the treatment of chronic neurodegenerative diseases of the visual and central nervous systems, such as glaucoma and Alzheimer's disease. Pharmacological efforts are required to prepare rhNGF for clinical trials and to identify the best route of administration.

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DISCLOSURES

The authors state no potential conflicts of interest.

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Table I. Potential use of NGF in ocular diseases.

Disease	Indications	Evidence
Corneal persistent epithelial defects	Neurotrophic keratitis	Experimental and clinical data (47-49)
Corneal ulcers and melting	Autoimmune diseases	Experimental and clinical data (51)
Infective keratitis	Herpes and postherpetic keratitis	Experimental data and clinical data (69, 70)
Keratoconjunctivitis sicca	After refractive surgery Sjögren's and non-Sjögren's dry eye	Experimental and clinical data (71) Experimental data (72, 73)
Cataract	Improve healing after surgery	Experimental and clinical data (52)
Cornea transplantation	Inhibit rejection	Experimental data (74)
Keratoconus	Trophic effect	Experimental data (75)
Optic nerve diseases	Traumatic, ischemic	Experimental data (8, 20)
Glaucoma	Neuroprotection	Experimental data (7, 21, 36, 37)
Retinal injury	Retinal detachment	Experimental data (22)
Degenerative retinal diseases	Retinitis pigmentosa	Experimental data (23, 24)
Diabetic retinopathy	Neuroprotection	Experimental data (25)

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EXHIBIT F

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Topical Nerve Growth Factor as a Visual Rescue Strategy in Pediatric Optic Gliomas : A Pilot Study Including Electrophysiology

Benedetto Falsini, Antonio Chiaretti, Giuseppe Barone, Marco Piccardi, Filomena Pierri, Cesare Colosimo, Ilaria Lazzareschi, Antonio Ruggiero, Vincenzo Parisi, Antonello Fadda, Emilio Balestrazzi and Riccardo Riccardi

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Abstract

Background. To date, no specific therapy is available for optic glioma (OG)–induced visual loss. *Objective.* To evaluate the effects on visual function of murine nerve growth factor (NGF) eye drop administration in children with severe visual impairment due to low-grade OGs. *Methods.* Five patients with OGs and advanced optic nerve atrophy were assessed before and after a single 10-day course of 1 mg murine NGF topical administration by clinical evaluation, visual evoked potentials (VEPs), and brain magnetic resonance imaging (MRI). VEPs, the main functional outcome measure, were recorded at baseline and 1, 30, 45, 90, and 180 days posttreatment. MRI examinations were performed at baseline and at 180 days after NGF treatment. Six untreated control patients with OGs also underwent serial VEPs, clinical testing, and MRI assessments. *Results.* After NGF treatment, median VEPs amplitude showed a progressive increase from the baseline values ($P < .01$). VEPs reached a maximum amplitude at 90 days (170% increase) and declined at 180 days, still remaining above the baseline level. Perception of spontaneous visual phosphenes was noted in all patients after NGF administration. MRI showed stable tumor size. In controls, clinical findings and VEPs did not show any significant change over the observation period. *Conclusions.* The findings from the study show that NGF administration may be an effective and safe adjunct therapy in children with optic atrophy due to OGs. The beneficial effect on optic nerve function suggests a visual rescuing mechanism exerted by murine NGF on the residual viable optic pathways.

Keywords

nerve growth factor, visual function rescue, optic gliomas, retinal ganglion cells, visual evoked potentials

Introduction

In children, several neoplastic diseases, such as craniopharyngiomas and optic gliomas (OGs), can compromise visual function involving both anterior and retrochiasmatic optic pathways. No specific therapy is currently available for OG-induced visual loss. Therefore, any improvement that might be obtained is of major clinical and socioeconomical value both to the patients and their relatives.

Nerve growth factor (NGF) is the first discovered neurotrophin involved in the development and survival of sympathetic, sensory, and forebrain cholinergic neurons.[1] In experimental animal models, NGF promotes nerve terminal outgrowth and neuron recovery after inflammatory, ischemic, and toxic injuries.[2,3] Given its actions favoring neuronal survival, NGF has been proposed for the treatment of some traumatic, ischemic, and neurodegenerative brain diseases.[4-6] Intraventricular NGF administration ameliorates symptoms in adults with Parkinson and Alzheimer disease and improves cerebral blood flow in infants with hypoxic-ischemic brain injury.[7-10] Exogenous NGF showed neuroprotective effects also on the visual system[11] due to the presence of NGF receptors on the conjunctiva, cornea, as well as in the retinal pigment epithelium, photoreceptors, and retinal

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Table 1. Clinical and Demographic Data of Patients Treated With Nerve Growth Factor and Controls

Patients Treated With NGF	Age, y, at Diagnosis, Sex	Age, y, at NGF Treatment	NF1	OG	COD	Previous Treatment S	Previous Treatment CT	MRI for Tumor Burden at 180 Days
1	0.8, Male	9.5	No	Yes	No	Yes	No	Stable
2	2.8, Female	16.4	Yes	Yes	No	No	Yes	Stable/regression
3	1.3, Male	4.6	Yes	Yes	No	No	Yes	Stable
4	4, Male	6.6	Yes	Yes	No	Yes	No	Stable
5	7.6, Female	18.5	No	Yes	No	Yes	Yes	Stable
Controls	**Age, y, at Diagnosis, Sex**	**Age, y, at Observation**	**NF1**	**OG**	**COD**	**S**	**CT**	**MRI for Tumor Burden at 180 Days**
6	4, Female	9.3	No	Yes	No	Yes	No	Stable
7	3, Male	16.2	No	Yes	No	No	Yes	Stable
8	3, Male	4.4	Yes	Yes	No	No	No	Stable
9	2.4, Male	6.5	Yes	Yes	No	No	No	Stable/progression
10	4, Male	18.3	No	Yes	No	Yes	No	Stable
11	2.8, Female	6.5	Yes	Yes	No	No	Yes	Stable

Abbreviations: NGF, nerve growth factor; NF1, neurofibromatosis; OG, optic glioma; COD, concomitant ocular diseases; S, surgery; CT, chemotherapy; MRI, magnetic resonance imaging.

ganglion cells (RGCs).[12] The effects of NGF and other neurotrophins, such as brain-derived neurotrophic factor (BDNF), are mediated via uptake by RGCs, anterograde transport along the optic nerve, and release to the postsynaptic geniculate neurons.[13]

In experimental animal models, intraocular NGF administration keeps RGCs from degeneration after optic nerve transection and ocular ischemia and leads to the rescue of axotomized forebrain cholinergic neurons,[14-16] while conjunctivally applied NGF proved to be effective in patients with corneal ulcers and severe glaucoma.[17,18] Indeed, it has been reported that NGF administered to the conjunctiva reaches rapidly the retina and the optic nerve where this neurotrophin exerts its biological activities through the uptake by RGCs.[19]

The aim of this study was to evaluate the safety and efficacy of NGF eye drops administration in improving optic nerve responses in advanced optic pathways damage induced by OGs. We report the treatment results of a pilot, open-label, longitudinal study in a group of 5 pediatric patients suffering from severe visual impairment associated with OG involving the chiasm and retrochiasmatic pathways. Results were compared with those obtained from an untreated control group of OG patients matched for age and disease severity.

Methods

Study Population

This open-label study was conducted in children with OGs without or with neurofibromatosis type I (NF1) by genetically confirmed diagnosis, admitted to the Division of Paediatric Oncology at the "Agostino Gemelli" Hospital in Rome, Italy. Five patients (3 males and 2 females) with severe impairment of visual acuity and visual field from optic nerve atrophy due to the presence of the tumor were included. None of the children enrolled had concomitant ocular diseases (Table 1). The median age was 9.5 years (mean age, 11 years; range, 4.6-18.5 years). Three patients had a diagnosis of NF-1. Before entering the study, 3 patients underwent 1 or more courses of chemotherapy (with an induction cycle of carboplatin and vincristine as suggested by Packer),[20] followed by maintenance treatment with the same drugs alternated with vincristine, 1-[2-chloroethyl]-3-cyclohexyl-1-nitrosurea] CCNU/procarbazine/desametasone.

At enrollment, all children were not receiving chemotherapy and the time interval between the last chemotherapy and the inclusion into the study was more than 24 months (mean time, 59.8 months; range, 25-131 months).

Six untreated control patients with OGs were also included in the study. They were matched for age and sex distribution, as well as for disease severity and residual visual function, to the treatment group.

Clinical and demographic features of all patients are reported in Table 1. Before starting treatment, children underwent a careful general and neuro-ophthalmologic examination. The latter included intrinsic ocular motility testing, voluntary conjugate eye movements assessment, anterior segment biomicroscopy, direct and indirect ophthalmoscopy, and clinical assessment of very low visual acuity ranging from light perception to hand motion. Given the very low vision condition of our patients, the visual acuity pretreatment

and posttreatment was considered as an unreliable outcome parameter, because of a possible bias in the examination. We therefore established, at the beginning of the study, to consider the visual evoked potentials (VEPs), recorded in response to steady-state flicker stimulation following a published technique,[21,22] as our main outcome measure in the evaluation of potential drug efficacy. All patients had clear optical media and no concomitant disorders that could have affected the electrophysiological assessment.

Cranial and orbital magnetic resonance imaging (MRI) scans were performed using standard imaging parameters and gadolinium enhancement. Initial and follow-up scans were reviewed by one neuroradiologist (CC) to evaluate the extent and location of tumor and to record any changes after the treatment. He did not know whether patients belonged to the control group or to the treatment group. OG location was determined as involving the chiasm and retrochiasmatic pathways in all patients. Changes in tumor size/volume were assessed by measuring the 3 largest diameters of the optic/hypothalamic gliomas. Sagittal and craniocaudal largest diameters were electronically calculated on sagittal T1-weighted postcontrast images; the largest transverse diameter was calculated on axial postcontrast T1-weighted images. In patients where the optic hypothalamic tumors showed partial contrast enhancement, the final values of the 3 largest diameters were defined using also unenhanced T2-weighted images. Moreover, the diameters of the intra-orbital optic nerves tumors were assessed on axial and coronal images. Finally—if present—the leptomeningeal metastatic deposits were also measured in terms of maximum thickness of the cisternal/leptomeningeal metastases.

All patients, both treated and controls, had participated in several repeated clinical, VEP, and MRI examinations before inclusion in the study. The clinical and VEP repeated sessions took place 2 to 4 months apart, and MRI evaluation 8 to 12 months. All patients were familiar either with the examination techniques that were employed in the study or with the personnel involved. This allowed reasonably good cooperation of all patients throughout the study. In addition, the data collected from the patients before inclusion in the study served as reference (eg, VEP test–retest variability) for comparison with the actual study data and provided a detailed picture of disease natural history for every patient.

All the enrolled patients (and/or their parents) were fully informed as to the nature and goals of the study. Written informed consent was obtained. The study followed the tenets of the Declaration of Helsinki and was approved by the ethics committee of the institution.

Visual Evoked Potential Methods

In all patients, electrophysiological testing was always performed within 1 week of the clinical and neuroimaging examinations by an independent examiner who was masked as to the patients' clinical and MRI findings or to the study group of NFG-treated or controls.

The stimulation/recording technique has been described in detail by Trisciuzzi et al.[21] Briefly, VEPs were recorded in response to the sinusoidal luminance modulation of a uniform field (temporal frequency = 8 Hz; modulation depth = 98%; mean luminance = 100 cd/m^2) generated by an array of 8 LEDs (peak emission wavelength 580 nm) presented monocularly in a mini-ganzfeld. During monocular stimulation (the right and left eyes were stimulated in a random order), the nonstimulated eye was kept adapted at the same mean luminance level as the stimulus. VEPs were recorded by using surface electrodes, placed on the scalp 3 cm above the inion and referenced to the right mastoid. The left mastoid was grounded. Interelectrode resistance was kept less than 5 kΩ. Signals were amplified (100 000 times), filtered (band-pass, 1-100 Hz), sampled with 12-bit resolution on a 100-μV AC range (2 kHz sampling rate), and averaged at the stimulus period (125 ms sweep) with automatic artifact rejection (±30 μV amplitude window). For each recording, 8 blocks of 200 sweeps were collected. A discrete Fourier series of the resulting grand means was performed off-line to isolate the fundamental (1F) component of the response, whose peak-to-peak amplitude (in μV) and phase angle (in degrees) were measured.[23] Since the response energy was mostly concentrated at this Fourier component, it may well represent the main VEP waveform for every record. The standard deviations (SDs) of individual blocks were measured to assess signal reliability. Typically, SDs were less than 30% for amplitude and 60° for phase. Responses were also averaged at a frequency 1.1 times the stimulus frequency to measure the residual "noise" level after averaging. Provided that noise spectrum is sufficiently smooth this gives a good estimate of noise amplitude at stimulus frequency. A "noise" evaluation was made using a control response at the same temporal frequency by presenting a subthreshold stimulus (ie, 0.001 modulation depth) whose mean luminance was the same as the actual stimulus. In both cases, the averaged noise at the 1F did not exceed 0.12 μV during all experimental sessions. At this noise level also baseline measurements that fall in the sub-microvolt region may be considered as reliable.

In both NGF-treated and control patients, potential retinal toxicity by concomitant chemotherapeutic drugs (or by NFG treatment in the former group) was excluded by ganzfeld scotopic and photopic electroretinogram testing performed according to ISCEV standards[24] at least twice during the observation period.

Nerve Growth Factor Isolation

The drug used was 2.5S NGF, which was purified and lyophilized from male mouse submandibular glands and prepared according to the method of Bocchini and Angeletti.[25] Briefly, the submandibular glands of adult male mice were explanted

under sterile conditions and the tissues were homogenized, centrifuged, and dialyzed. This aqueous gland extract was then passed through subsequent cellulose columns, thereby separating NGF by adsorption. The first step was gel filtration at pH 7.5, in which most of the active NGF was eluted in the 80 000 to 90 000 molecular weight range. The samples obtained were analyzed by spectrophotometry at a wavelength of 280 nm to identify NGF-containing fractions. Specificity of fractions was determined by Western blot analysis. NGF purity (>95%) was estimated by high-performance liquid chromatography, the column equipped with a guard column calibrated with 40 mg of purified and bioactive murine 2.5S NGF standard. The NGF obtained was then dialyzed and lyophilized under sterile conditions and stored at −20°C until used. Biological activity of purified NGF was evaluated by in vitro stimulation of neurite outgrowth in rat pheochromocytoma PC12 cells over a period of 7 to 14 days. Subsequently, NGF was dissolved in 0.9% sterile saline solution in concentrations of 200 μg/mL. The concentration of NGF in this solution was stable for the entire treatment time.

Nerve Growth Factor Eye Drop Administration

NGF eye drop was administered after at least 2.5 years from the diagnosis of OGs, if the patients showed loss or severe impairment of visual acuity. A total of 1 mg of NGF diluted in 5 mL of saline solution was administered onto the conjunctiva of both eyes for 10 consecutive days 3 times a day. This amount is considered sufficient to reach and stimulate NGF receptors in most cerebral cholinergic areas of the brain and optic pathways, as previously reported in the literature.[7] We preferred to use murine NGF, instead of human-recombinant NGF, because contrasting results have been reported on the efficacy of the latter, mainly due to a lack of in vivo studies. Clinical studies using human NGF have been performed only in patients with sensory polyneuropathy reporting either positive effects[26] or no significant effects on this condition.[27] Currently, there are no studies on conjunctivally applied human NGF, unlike that reported for murine NGF demonstrating a therapeutic effect for corneal pathologies[17] or supporting a potential therapeutic effect for retinal degenerations.[18] In addition, there is no evidence showing that the human NGF is able to reach and affect the optic nerve and brain areas, such as the septum and Meynert basal nuclei, as reported for murine NGF.[19,28]

Follow-up and Testing Schedule

In both groups of patients (the NGF treated and the controls), clinical, VEP, and MRI examinations were performed at the beginning of the follow-up period. In the treatment group, clinical and VEP testing were repeated at 10, 30, 45, 90, and 180 days after the beginning of the NGF treatment. MRI was repeated at 180 days. In the control group, clinical and VEP examinations were repeated at the same time points throughout the observation period. MRI was repeated at 180 days.

Ocular and Systemic Complications Potentially Related to NGF Administration

During the entire period of assessment (180 days; see below) particular attention was paid to detect ocular and/or systemic side effects. Potential ocular complications included inflammation (external or on the iris and/or ciliary body), pain, development of lens opacities, and increased intraocular pressure. Systemic complications included acutely increased intracranial pressure and development of acute intratumor hemorrhagic infarction, as well as allergic reactions. Progression of the OG mass, as reflected by a volumetric increase of more than 25% from baseline at brain MRI, was regarded as a potential adverse outcome event. Other potential side effects related to NGF administration were systemic pain as well as weight loss, as previously reported in the literature.[7]

Statistical Analysis

VEP amplitude and phase results were evaluated in both treated and control patients by nonparametric statistics. Data obtained over the follow-up period were statistically analyzed using a method that takes into account the circular distribution of phase space after conversion of amplitude and phase data into cosine and sine values.[29] Longitudinal changes (baseline vs end of follow-up) of VEPs from OGs patients were evaluated by statistically comparing (by means of nonparametric analysis of variance [ANOVA]) the amplitude and phase values across the recording sessions. Percentage amplitude difference (ie, 2nd − 1st test/1st test × 100) and phase difference in degrees (taking into account circular phase distribution) between test results were calculated for each patient and the median, 5th, and 95th percentiles of the resulting distributions established. In some analyses, VEP amplitudes were also converted to log 10 values to better approximate normal distribution. In all the analyses, a P value <.05 was considered as statistically significant.

Results

In treated patients, no local or systemic adverse events related to NGF treatment were observed either during treatment or over the 180-day period of follow-up.

At baseline, in all patients the visual acuity was extremely low, ranging from light perception to hand motion perception at the distance of 10 cm. Pupils were normal in shape and size (mean pupil size, 4 mm; range, 3.5-5 mm). Ganzfeld

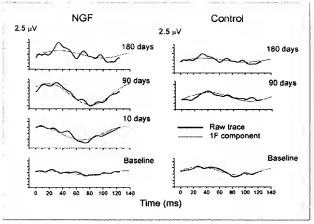

Figure 1. Representative visual evoked potential waveforms recorded in a nerve growth factor (NGF)–treated patient at baseline, 10, 90, and 180 days. For each record, the Fourier-analyzed fundamental harmonic component is also shown (in grey). Data from a control patient are shown at baseline, 90, and 180 days of follow-up. For each record, the sweep duration is equivalent to 1 stimulus cycle (8 Hz, 125 ms).

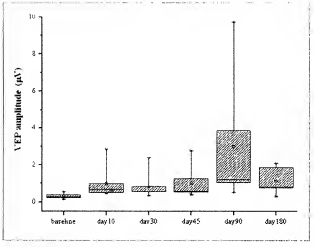

Figure 2. Box plots showing the distribution of visual evoked potential (VEP) 1F amplitude values recorded in nerve growth factor (NGF)–treated patients at baseline and at various times following NGF treatment. Each box shows 75th, 50th, and 25th percentiles. The point inside the box indicates the mean. Error bars show 99th and 1st percentiles.

electroretinograms (ERGs) were normal in both amplitude and implicit time. This allowed us to exclude any potential retinal damage due to the previous chemotherapeutic treatment.

After NGF treatment and during the 180-day period of follow-up, patients and/or parents on their behalf reported some visual improvements during and after the treatment. All of them reported phosphene perception at night, and some figures' shapes instead of light only in the daylight. In particular, a patient (case 2 in Table 1) started watching TV using an anomalous head posture. Subjective symptoms of visual improvement were never reported in the untreated controls during the follow-up. No changes in pupil size or pupillary light response were detected after NGF treatment.

The flicker VEPs results are shown in Figures 1 to 3. In Figure 1, representative VEP waveforms recorded in an NGF-treated patient at baseline, 10, 90, and 180 days are shown.

The response fundamental component, shown in grey, isolated by Fourier analysis, is also reported in the figure for each record. Data from a control patient are shown at baseline, 90, and 180 days of follow-up. It can be noted that in the treated patient, but not in the control, the VEP response showed a clear increase in amplitude at both 90 and 180 days compared with baseline.

In Figure 2, box plots showing the distribution of VEP amplitude values recorded in NGF-treated patients at baseline and at various times following treatment are reported. Each box shows 75th, 50th (median), and 25th percentiles. The point inside each box indicates the mean. Error bars show 99th and 1st percentiles. In all treated children, median VEP amplitude, severely attenuated at baseline compared with normal values,[22] showed a sharp increase immediately after the end of the treatment. This improvement lasted throughout the entire follow-up period, progressively reaching a maximum after 90 days (170%) and declined afterward, but still remained above the posttreatment values. The changes shown in Figure 2 were statistically significant by nonparametric, repeated-measures ANOVA ($P < .01$). No statistically significant changes in the VEP phase of the treated patients were found throughout the follow-up. In the untreated controls, no significant changes in VEP amplitude and phase were found throughout the follow-up. The magnitude of VEP amplitude changes found during the follow-up in the treated patients was significantly greater than that observed in the test–retest assessment either in the untreated controls or in the treated patients before inclusion in the study (see Methods).

Table 2 reports the variability of VEP measurements obtained in 2 VEP recording sessions recorded 6 months

Table 2. Test–Retest Variability Measurements for VEP 1F Component Recorded in Control Patients Before Inclusion in the Study

	VEP 1F Amplitude (μV)		
	1st Test	2nd Test	% Difference[a]
Median	0.66	0.71	7.45
5th percentile			−55.47
95th percentile			51.08

Abbreviation: VEP, visual evoked potential.
[a]Percentage difference in amplitude (μV) between second and first tests.

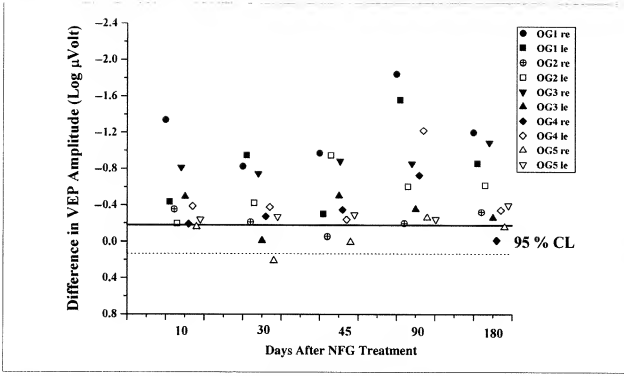

Figure 3. Scatterplots showing the individual visual evoked potential (VEP) log amplitude changes from baseline (ie, log amplitude at a given recording session minus log amplitude at baseline) observed in each eye of all participants at the different follow-up times. Horizontal lines in the plot indicate the 95% confidence limits for test–retest amplitude changes observed in the same patients before inclusion in the study. NGF, nerve growth factor.

apart in the untreated controls. It can be noted that the difference within tests is smaller and divided between changes above or below the baseline level, as compared with the changes observed in treated patients after NGF treatment.

These last findings are shown in Figure 3, which depicts, as scatterplots, the individual VEP log amplitude changes from baseline (ie, log amplitude at a given recording session minus log amplitude at baseline or log amplitude ratio) observed in each eye of all participants at the different follow-up times. Horizontal lines in the plot indicate the 95% confidence limits for test–retest amplitude changes observed in the same patients before inclusion in the study. VEP amplitude increased following treatment in most of the recordings, indicating that the general trend toward an improvement was observed in all individual patients. In addition, in most of the recording sessions the VEP amplitude increase was higher than the 95% confidence limits established for test–retest variability observed in the same patients before the study. MRI evaluation, performed at baseline and after 180 days in the treated and untreated children, did not show any significant change in tumor size and volume after NGF treatment. Both morphometric and morphological evaluations on the optic pathways after 180 days did not show any changes compared with the baseline (Table 1).

Discussion

We evaluated safety and efficacy of NGF, administered via the conjunctiva, as a potential neuroprotective factor for severely damaged optic pathways in children suffering from OGs. Although spontaneous regression of optic pathway gliomas associated with significant visual improvement may occur in rare patients,[30] we are unaware of any cases in which children with vision in the range of our patients spontaneously improved. Thus, we do not believe that the clinical and electrophysiologic improvement observed in our patients were spontaneous, but on the contrary, we suppose that this improvement was related to the signaling effect on damaged but still viable RGCs exerted by NGF. This effect resulted in an increased visual response from these cells.

Our study demonstrated that in treated patients the functional electrophysiological results showed a significant improvement of the flicker-evoked VEP amplitudes following NGF administration. The magnitude of such improvement was far greater than the test–retest variability observed either in treated patients before their enrolment or in untreated controls. The prolonged and sustained time course of VEPs amplitude changes following a short NGF treatment course (10 days) suggests a sequence of molecular events into the surviving RGCs leading to a recovery of their physiological responses.

It is known that RGCs express NGF receptor (TrkA) and that NGF, binding to TrkA, upregulates Bcl-2 protein, which protects cells from apoptosis by preventing caspase activation.[31,32] Furthermore, intravitreal NGF delivery to the retina and optic nerve is crucial to the survival of RGCs and for functional recovery of the retina following ocular ischemia and hypertension in experimental animal models.[15,33] Last, conjunctivally applied NGF has been shown to reach sharply the retina and optic nerve where this neurotrophin exerts its biological activities.[19]

Ten days of eye drop NGF administration in 5 blind or severely visually impaired children resulted in a significant improvement in VEP responses. Despite any attempt to control the effects of tumor progression on optic pathways by medical and chemotherapy treatment, these patients had progressive visual loss and severe abnormalities in their VEP responses. These electrofunctional abnormalities reflected a dysfunction of the innermost retinal layers, delay in visual cortical responses, and delay in neural conduction along postretinal visual pathways related to RGCs and optic nerve degeneration. The significant improvement of VEPs observed in NGF-treated patients suggests a functional recovery of RGCs and an improvement of neural conduction along the postretinal visual pathways. These effects confirm the key role of neurotrophins in modulating RGCs function and visual cortical neuronal activity reflected by receptive field size and response latency.[34-36]

Improvement of VEP amplitude persisted for about 180 days after discontinuation of treatment, indicating that changes induced by NGF had a prolonged duration. This prolonged and stable NGF effect may be related not only to a protective activity against neural apoptosis but also to the formation of new neural pathways, since it is known that NGF promotes neuronal repair and axonal regeneration.[37-39] NGF acts on different levels to promote neuronal recovery following ischemic, inflammatory, and traumatic injuries: through a neosynaptogenetic mechanism, by directly affecting precursor cells, and/or by induction of other growth factors, such as BDNF, whose neuroprotective effects on visually evoked RGC responses after optic nerve section has been reported.[13,40-43] These different neuroprotective mechanisms exerted by NGF may cause the significant improvement of VEP response observed during and after NGF treatment.

In our patients, the electrofunctional changes of VEPs after NGF administration were also followed by some clinical ameliorations, without any systemic or ocular side effects. Reporting on subject improvements was difficult to obtain in these subsets of patients since their residual sight was almost null. Nevertheless, we asked them or their parents to report on any new visual sign or behavior attributable to the improved visual function. Different from untreated controls, who never reported subjective symptoms of visual improvement during the follow-up period, all children treated with NGF had phosphenes perception at night, reporting figures' shapes instead of light only, and, in 1 case, attempting watching TV. In this last case, we assumed the child was trying to involve a part of the peripheral temporal visual field that was still sensitive to visual stimuli. All these signs could be considered as indicators of partial visual recovery after NGF administration, supporting the hypothesis that conjunctivally applied NGF can reach easily the retina and the optic nerve where this neurotrophin exerts its neuroprotective effects on residual viable optic pathways, as previously reported both in experimental animal models and in adult patients with severe glaucoma.[18,19]

In conclusion, this is a first step toward the development of a large clinical project aimed at evaluating the potential effectiveness of NGF eye drop administration for improving visual function in patients with low-grade OGs affecting the optic pathways. The current preliminary findings and the ease of administration of the drug make it worthwhile to be investigated further, mainly in OG patients with better baseline visual functions, in order to explore more thoroughly the benefits of NGF on visual function recovery. Although further controlled, randomized, double-blind studies are needed for a better understanding of the neuroprotective mechanisms of this neurotrophin, eye drop NGF administration appears to be a promising rescuing strategy for the treatment of children with different neurodegenerative diseases that involve the optic pathway.

Authors' Note

Benedetto Falsini and Antonio Chiaretti contributed equally to this study.

Declaration of Conflicting Interests

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Case Report

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Abstract

Background. Spontaneous visual improvement in people with an optic glioma (OG) of the anterior or retrochiasmatic optic pathways is rare. *Objective.* To evaluate the effects on visual function of nerve growth factor (NGF) eye drop administration in a patient with severe visual impairment due to a low-grade OG. *Methods.* A 45-year-old woman with OG and long-standing optic nerve atrophy was assessed before and after 2 NGF treatment courses. The drug used was 2.5S murine NGF. One milligram of NGF, diluted in saline solution, was administered onto the conjunctiva of both eyes for 10 consecutive days 3 times a day for each treatment. The follow-up was performed by clinical, neuroradiologic, and electrophysiological tests (electroretinogram and visual evoked potentials [VEPs]) at the end of each treatment and 30 and 60 days later. *Results.* A repeated subjective and objective improvement of visual function (>3 lines visual acuity; >40° visual field; >50% VEP amplitude increase, Wilcoxon test $P < .01$) was recorded after NGF treatment. These measures tended to deteriorate toward baseline values 60 days from the end of each NGF treatment. No ocular or systemic side effects were observed throughout treatment. *Conclusions.* Conjunctival NGF may be a beneficial adjunct therapy for visual loss in patients with OG, possibly exerting its effects on residual viable optic pathways.

Keywords

nerve growth factor, neuroprotection, optic gliomas, vision loss

Introduction

Several neoplastic diseases, such as craniopharyngioma and optic glioma (OG), can compromise visual function involving both anterior and retrochiasmatic optic pathways. No specific therapy is currently available for OG-induced visual loss. Nerve growth factor (NGF) is the first discovered neurotrophin involved in the development and survival of sympathetic, sensory, and forebrain cholinergic neurons.[1] In experimental animal models, NGF promotes nerve terminal outgrowth and neuron recovery after inflammatory, ischemic, and toxic injuries.[2] Given its actions favoring neuronal survival, NGF has been proposed for the treatment of some traumatic, ischemic, and neurodegenerative brain diseases.[3,4] Intraventricular NGF administration may ameliorate symptoms in adults with Parkinson's and Alzheimer's disease and improves cerebral blood flow in infants with hypoxic–ischemic brain injury.[5-8] Exogenous NGF showed neuroprotective effects also on the visual system[9] because of the presence of NGF receptors on the conjunctiva, cornea, and retinal ganglion cells (RGCs).[10] The effects of NGF and other neurotrophins, such as brain-derived neurotrophic factor (BDNF), are mediated via uptake by RGCs, anterograde transport along the optic nerve, and release to the postsynaptic geniculate neurons.[11] In experimental animal models, intraocular NGF administration keeps RGCs from degeneration after optic nerve transection and ocular ischemia and leads to the rescue of axotomized forebrain cholinergic neurons,[12,13] whereas conjunctivally applied NGF proved to be effective in patients with corneal ulcers and severe glaucoma.[14,15] Indeed, it has been reported that NGF administered to the

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conjunctiva rapidly reaches the retina and the optic nerve where this neurotrophin exerts its biological activities through the uptake by RGCs.[16] Based on these evidences, we report the first known case of NGF effect on visual loss reversal in a patient with OG.

Case Report

A 45-year-old woman, affected since childhood by genetically confirmed neurofibromatosis type I, had a diagnosis of anterior prechiasmatic OG at age 33 years with bilateral and steadily progressive deterioration of both central and peripheral vision. At admission neuro-ophthalmologic examination showed bilateral proptosis of 3 mm, normal extrinsic and intrinsic ocular motility. Best corrected visual acuity was as follows: right 0.2 (−6 sph), left 0.1 (−8 sph); near distance: 5th size (Jaeger reading chart) bilaterally. Ishihara plate testing showed bilateral red-green axis dyschromatopsia. Goldmann visual field testing showed bilateral concentric, severe constriction with both V/4e and II/4e isopters (main diameter = 15° and 10°, respectively). Visual evoked potentials (VEPs) pattern reversal and flicker (8 Hz) stimulation showed severe bilateral amplitude attenuation with an increased time-to-peak. Electroretinograms (ERGs) showed attenuated amplitudes bilaterally of the inner retina–related flicker 1st and 2nd harmonic components.

Based on the role of NGF in important biochemical and molecular mechanisms of the optic pathways, we decided to treat this patient by NGF eye drop administration when marked and progressive visual loss was no longer responsive to conventional treatment with steroids and mannitol. The second NGF course was carried out after 60 days in the same fashion, according to an institutional review board/ethical committee–approved protocol.

The drug used was 2.5S NGF, purified and lyophilized from male mouse submandibular glands and prepared according to the method of Bocchini and Angeletti.[17] The submandibular glands of adult male mice were explanted under sterile conditions and the tissues were homogenized, centrifuged, and dialyzed. This aqueous gland extract was then passed through subsequent cellulose columns, thereby separating NGF by adsorption. The first step was gel filtration at pH 7.5, wherein most of the active NGF was eluted in the 80 000 to 90 000 molecular weight range. The samples obtained were analyzed by spectrophotometry at a wavelength of 280 nm to identify NGF-containing fractions. Specificity of fractions was determined by Western blot analysis. NGF purity (>95%) was estimated by high-performance liquid chromatography column equipped with a guard column calibrated with 40 mg of purified and bioactive murine 2.5S NGF standard. The NGF obtained was then dialyzed and lyophilized under sterile conditions and stored at −20° until used. Biological activity of purified NGF was evaluated by in vitro stimulation of neurite outgrowth in rat pheochromocytoma PC12 cells over a period of 7 to 14 days. NGF was dissolved in 0.9% sterile saline solution in concentrations of 200 μg/mL. The concentration of NGF in this solution was stable for the entire treatment time. One milligram of NGF, diluted in 5 mL of saline solution (0.2 mg/mL), was administered onto the conjunctiva of both eyes for 10 consecutive days 3 times a day for each treatment. This dose is considered sufficient to reach and stimulate NGF receptors in most cerebral cholinergic areas of the brain and optic pathways, as previously reported in the literature.[5]

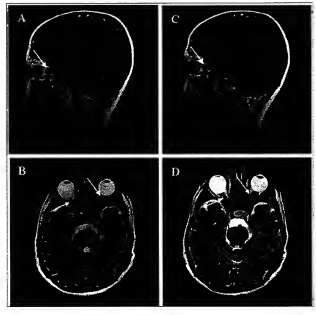

Figure 1. Sagittal T1-weighted magnetic resonance imaging scan (MRI) of the brain and orbits showing the glioma affecting the left optic nerve (white arrow) at baseline (A) and 4 months after nerve growth factor (NGF) administration (C). Axial T2-weighted MRI showing both right and left optic nerves involved by bilateral optic gliomas (white arrow) at baseline (B) and 4 months after NGF administration (D). No significant change in tumor volume after NGF treatment was found

No ocular or systemic side effects occurred. Brain magnetic resonance imaging (MRI) evaluation, performed at baseline and after 120 days, did not show any significant change in tumor volume after NGF treatment (Figure 1 A-D). The follow-up was performed by clinical and electrophysiological tests at the end of each treatment and after further 30 and 60 days. A subjective and objective improvement of visual acuity and visual field (>3 lines visual acuity; >40° visual field; Wilcoxon test, $P < .01$; magnitude of change >99 percentile of the test–retest variability for both visual acuity and visual field[18,19]; Figure 2A and B) allowing her to walk confidently, watch TV, and recover the customary activities was recorded after 48 hours from the

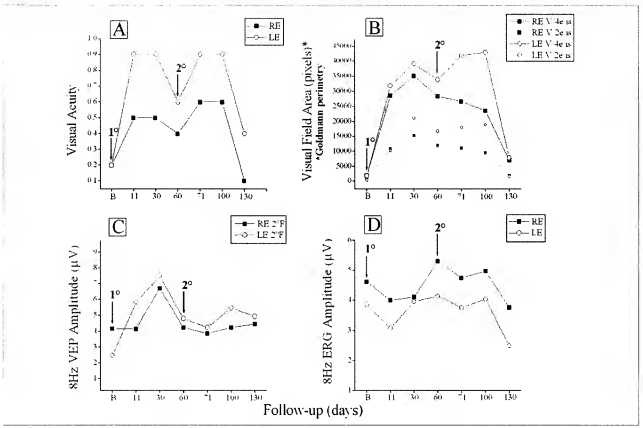

Figure 2. Right after the end of each treatment, visual acuity (A) recovered in both eyes by 3 to 7 lines (significant [$P < .05$] increase or decrease is ≥2 lines). (B) Lateral visual fields reported in relative units following planimetric analysis, recovered by several log units of area indicating a main increase in lateral perception of 40° in RE and 35° in LE. (C) VEPs showed an increase in amplitude in parallel with visual acuity improvement. (D) ERGs showed amplitude enhancement 60 days after the first NGF course and then tended to decline

Abbreviations: B, baseline; ERG, electroretinogram; LE, left eye; NGF, nerve growth factor; 1°, first NGF administration; 2°, second NGF administration; RE, right eye; VEP, visual evoked potential.

beginning of each treatment. Also, VEPs and ERGs showed an improvement after NGF administration (>50% increase in VEP amplitude; changes >99 percentile of the test–retest variability[20]; Figure 2C and D). Visual acuity, visual field, and VEPs tended, following the improvement, to deteriorate toward baseline values after 60 days from the end of each NGF treatment, suggesting the need to continue NGF administration every 2 months.

Discussion

This report shows that NGF eye drop administration may ameliorate visual acuity, visual field, VEPs, and ERGs amplitudes in this patient with OG, as indicators of visual loss reversal after NGF treatment. The improvement of VEPs and ERGs suggests molecular events within surviving RGCs leading to a recovery of their physiological responses. It is known that RGCs express NGF receptor (TrkA) and that NGF, binding to TrkA, upregulates Bcl-2 protein, which protects cells from apoptosis by preventing caspase activation.[21] Furthermore, intravitreal NGF delivery to the retina and optic nerve is crucial to the survival of RGCs and for functional recovery of the retina following ocular ischemia and hypertension in experimental animal models.[13,22] Last, conjunctivally applied NGF has been shown to reach sharply the retina and optic nerve where this neurotrophin exerts its biological activities.[16] In our patient, the electrofunctional changes after NGF administration were immediately followed by clinical improvement. These effects confirm the key role of neurotrophins in modulating RGC function and visual cortical neuronal activity, as reflected by receptive field size and response latency.[23,24] Amelioration of both clinical and electrophysiological parameters lasted for about 60 days after NGF treatment, indicating that NGF effect on optic pathways had a prolonged duration. This prolonged and stable NGF effect may

be related not only to a protective activity against neural apoptosis but also to the formation of new neural pathways, since it is known that NGF promotes neuronal repair and axonal regeneration.[25,26] NGF acts on different levels to promote neuronal recovery following ischemic, inflammatory, and traumatic injuries: through a neosynaptogenetic mechanism, by directly affecting precursor cells, and/or by induction of other growth factors, such as BDNF, whose neuroprotective effects on visually evoked RGC responses after optic nerve section have been reported.[11,27]

Caution is necessary. NGF promotes proliferative activity of glioblastoma cells through direct interaction with activation of RAS and MAPK Erk1/2 pathway.[28] The level of NGF increases in the malignant glioma tissue,[29] although the opposite has been found for low-grade astrocytomas,[30] whereas growth control of glioma by NGF has been shown in vivo.[31] Currently, drugs that block RAS and mTOR are under investigation for treatment of NF1-associated malignancies (see www.clinicaltrials.gov). Potential complications of long-term therapy using topical NGF treatment on NF1-associated OG include an increase in tumor size. A recent 6-month, phase I trial of topical NGF in children with OG from our group did not show any growth by MRI.

Prospective and controlled clinical trials should be conducted to explore the efficacy of conjunctivally applied NGF on optic nerve function. In parallel, animal studies could identify NGF-induced mechanisms in genetically engineered mice with knockout of the NF-1 gene that develop OG.[32] These animal models could evaluate the changes and correlations between NGF and other neurotrophic factors, such as BDNF, basic fibroblast growth factor, ciliary neurotrophic factor, and synaptic vesicle modifications.

Authors' Note

Antonio Chiaretti and Benedetto Falsini contributed equally to this study.

Declaration of Conflicting Interests

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Nerve growth factor eye drops improve visual acuity and electrofunctional activity in age-related macular degeneration: a case report

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Summary. Age-related macular degeneration (ARMD) is a severe disease affecting visual function in the elderly. Currently available surgical and medical options do not guarantee a significant impact on the outcome of the disease. We describe the effects of nerve growth factor eye drop treatment in a 94 years old female with ARMD, whose visual acuity was progressively worsening in spite of previous surgical and medical treatments. NGF eye drops improved visual acuity and electrofunctional parameters as early as 3 months after initiation of treatment. These results are in line with previous reports on a neuroprotective effect of NGF on retinal cells and on NGF eye drops bioavailability in the retina and optic nerve. No side effects were observed after five years of follow-up, suggesting that topical NGF treatment may be a safe and effective therapy for ARMD.

Key words: macular degeneration, nerve growth factor, visual pathways, retina, eye drops.

Riassunto *(Il fattore di crescita nervosa in collirio migliora l'acuità visiva e l'attività elettrofunzionale nella degenerazione maculare senile: un case report).* La degenerazione maculare senile (DMLE) rappresenta una patologia della terza età ad elevato impatto sociale che determina una grave riduzione della funzione visiva. Attualmente non vi sono terapie mediche o chirurgiche sicuramente in grado di arrestarne la progressione. In questo studio descriviamo l'effetto della somministrazione di NGF in collirio in una paziente di 94 anni affetta da DMLE, con un quadro clinico progressivamente ingravescente nonostante fosse stata sottoposta ad interventi chirurgici. Il trattamento con NGF ha determinato un miglioramento dell'acuità visiva e dei parametri elettrofunzionali retinici già dopo 3 mesi. Questo risultato è in linea con dati sperimentali che dimostrano come l'NGF in collirio raggiunga la retina ed il nervo ottico, dove svolge un ruolo neuroprotettivo. Gli effetti benefici dell'NGF sono perdurati per tutto il periodo di follow-up (6 anni) in assenza di effetti collaterali. Questo dato suggerisce la possibilità di un utilizzo terapeutico dell'NGF in collirio per patologie degenerative retiniche.

Parole chiave: degenerazione maculare, fattore di crescita nervosa, vie ottiche, retina, gocce oftalmiche.

INTRODUCTION

A consistent number of studies published in recent years have demonstrated that topical administration of nerve growth factor (NGF) to the eye is a safe therapy able to restore corneal integrity in patients with neurotrophic ulcers [1-3]. The healing properties of NGF have also been reported in cutaneous ulcers caused by diabetes, rheumathoid arthritis and pressure ulcers [4-9]. More recently, studies in animal models of ocular hypertension and glaucoma have shown that retinal cells express NGF receptors and that the degeneration of these cells can be protected by NGF eye drop administration [10, 11]. Based on these data we hypothesized that topical NGF administration to the eye could be a novel therapeutic approach for human macular degeneration.

Age-related macular degeneration (ARMD) is the most common cause of irreversible visual loss in the elderly in the developed countries, and its incidence is constantly increasing [12]. Loss of vision due to ARMD occurs either as a result of choroidal neovascularisation with exudation and haemorrhage ("wet" ARMD) or by slow atrophy of the retinal-pigment epithelium and overlying receptors ("dry" ARMD) [13]. Definitive treatment does not yet exist. About 10% of patients with wet ARMD who present early with distorted vision and an eccentric neovascular lesion can be treated with laser photocoagulation. However, the recurrence rate is over 60%, with further

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vision being usually lost [14]. Selected patients can also be treated with photodynamic therapy involving low-intensity red laser treatment after intravenous verteporfin infusion, but the functional benefit is usually modest [15]. In the last few years, treatment with repeated intravitreal injections of anti-angiogenic compounds has also been proposed, with substantial success in reducing progression of disease [16, 17]. Nevertheless, several alternative pharmacological interventions are in different phases of clinical development, and researchers are guardedly optimistic that these advances may change the entire approach to ARMD management in the near future.

Based on our long-lasting experience on the healing action of NGF in corneal ulcers, and the evidence that this form of NGF application does not exert side effects or generate circulating NGF antibodies [1], the possible use of topical NGF in ARMD was taken into consideration. The effect of eye NGF treatment was valuated in a 94 years old female patient affected by maculopathy for over three years characterized by progressive worsening of visual acuity and macular degeneration as evidenced by clinical and electrofunctional tests. Moreover, since all available therapies failed to arrest or delay the visual field worsening induced by the maculopathy, informed signed consent was obtained and the patient was treated TID with NGF eye drops. The effects of therapy were followed closely every-other day for the first three weeks and every 3 months thereafter.

The results of this case report are based on the evaluation of visual acuity and electrofunctional parameters during 6 years of NGF treatment.

MATERIALS AND METHODS

A 94 years old female affected by bilateral wet ARMD since 1995 referred to our Department following macular cryotreatment in left eye (in 1996), and laser and photodynamic treatment in right eye (in 2000 and 2001, respectively). Cataract surgery was performed in both eyes (in 2001). Eye examination showed the presence of bilateral macular scar with no evidence of neovascularisation (*Figure 1*). Fluorescein examination confirmed a central extensive area of retinal atrophy with the absence of choroidal neovascularization. Best corrected visual acuity (BCVA) was 0.05 in right eye and count finger in left eye. J17 was the near visual acuity detectable in the right eye, while it was not detectable in the left eye. At baseline, electrofunctional exams showed a decrease of ERG amplitude (*Figure 2*).

After all currently available therapies failed to block or delay the visual deficits induced by ARMD progression, the patient signed informed consent to be treated with NGF eye drop on a compassionate basis. Highly purified NGF was prepared following the Bocchini&Angeletti method, as reported in our previous studies. [3. 8, 18] Lyophilized NGF was dissolved in physiological saline solution and one drop (approximately 50 µl) of 200 µg/ml was administrated TID into the conjunctival fornix of the right eye.

Fig. 1 | Fundus oculi *examination by indirect ophthalmoscopy evidenced the presence of a bilateral macular scar (arrow). A photograph taken from right eye before initiation of NGF eye drop treatment is shown.*

RESULTS

The patient was evaluated at baseline and every three months during NGF treatment and the follow-up (6 years) by complete ocular examination including BCVA and electrofunctional exams. No ocular adverse side effects were observed during the treatment and follow-up. Macular morphology was unchanged at the end of follow-up. However, after 3 months of NGF treatment an improvement of ERG

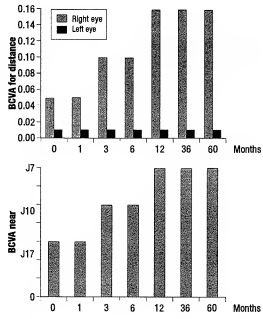

Fig. 2 | *Topical NGF treatment in the right eye improved ERG amplitude, while no improvements were observed in the left (untreated) eye.*

amplitude was observed (*Figure 2*), followed by an increase of visual acuity in the right eye that reached 0.16 and J7 after 1 year of treatment. No changes were observed in the left eye.

At 1 year of NGF treatment, patient discontinued NGF eye drop for 3 months. Interestingly, the follow-up visit at 3 months of NGF discontinuation showed decreased ERG amplitude, associated with a decreased visual acuity. Thus, patient re-started NGF eye drop treatment and after 3 months of treatment ERG amplitude and visual acuity increased again. At 18 months of NGF treatment both BCVA and ERG value were stable. No morphological changes were observed in the retina of both eyes.

The only side effect recorded during the 6 years of NGF treatment was a weak burning sensation at the time of administration that persisted for the 1st month of treatment.

DISCUSSION

Nerve growth factor (NGF) is an endogenous neurotrophin that exerts trophic and differentiative activity on neurons of the central and peripheral nervous systems with protective and/or regenerative effects observed in degenerative diseases or following injury [6, 19-23]. Intraocular administration of NGF in animal models has been shown to inhibit retinal ganglion cell degeneration [24, 25]. Despite the numerous data showing NGF abilities in protecting and recovering retina and optic nerve from injury and/or degeneration, invasive routes of administration, such as intraocular injection, or intraocular insert releasing the growth factors, are not optimal for clinical use. Recently, NGF applied topically to the eye has also been shown to reach the retina, optic nerve and brain [26, 27], indicating that NGF eye drops could be a novel therapy for the treatment of ocular and brain degenerative diseases.

In this study, we report the case of a patient with advanced bilateral ARMD who improved both her BCVA and electrofunctional parameters with TID topical NGF administration in one eye for 5 years, without reporting any side effect except for a mild and transient burning sensation at the site of administration for one month after initiation of treatment.

The efficacy of NGF in improving visual acuity and electrofunctional parameters has been observed in this patient at 3 months after initiation of treatment. This "early" effect of NGF treatment is in line with the NGF action in supporting trophism and functions of central and peripheral neurons *in vitro* as well as *in vivo* [22, 28, 29]. Specifically, several studies have demonstrated a biological activity of NGF in protecting retinal ganglion cells against ischemic, traumatic and hypertensive injuries [24, 25, 30]. This neuroprotective properties of NGF make it a strong candidate for the future treatment of degenerative diseases (such as glaucoma). In fact, it is also noteworthy that in the course of NGF eye drops treatment or during the follow up, no NGF antibodies were detected in serum, as the murine NGF presents great homology with human NGF and the amount of topically-applied NGF reaching the bloodstream is absent or too low to trigger anti-NGF-antibodies formation [1].

A key question raised by this clinical observation and also by studies on animal models [10, 11, 27, 31], is how NGF, a high-molecular-weight protein, is transported from the ocular surface to the posterior segment of the eye when administered topically as eye drops. The evidence currently available points out to two possibile routes: through the cornea, conjunctiva, sclera, choroid, choriocapillaris, and retinal pigment epithelium, or indirectly by traveling through the retrobulbar space to the optic nerve.

Although the clinical observation presented in this report needs to be confirmed on a large scale clinical trial, the good clinical response and the persisting of the improvement during the follow-up stimulate to extend our pilot clinical study. Moreover this study confirms the possibility of using the eye drop NGF for reaching retina, optic nerve and brain in a safe and non-invasive manner, supporting the potential use of topical NGF therapy for ocular and brain degenerative diseases currently considered untreatable.

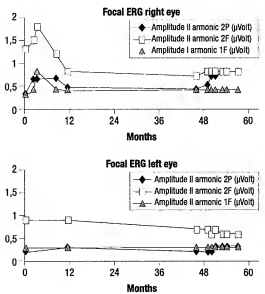

Fig. 3 | *Topical NGF treatment improved best corrected visual acuity (BCVA) both for near and distance in the right eye as early as three months after initiation of therapy. Visual acuity in the right eye reached 0.16 and J7 after 1 year of treatment. No changes were observed in the left (untreated) eye.*

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EXHIBIT I

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Reduced NGF level and TrkA protein and TrkA gene expression in the optic nerve of rats with experimentally induced glaucoma

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ABSTRACT

Glaucoma (GL) is an optic neuropathy characterized by progressive loss of visual field due to retinal cell death and optic nerve (ON) degeneration, usually in response to abnormal elevated intraocular pressure (EIOP). It has previously demonstrated that cells of the ON express nerve growth factor (NGF) and NGF-receptors. Relatively little is known, however, about their role on the ON of the glaucomatous eye. The aim of the study was to elucidate this aspect. Using a rat model of GL we investigated the response of NGF and NGF-receptors in the ON of subjects with experimentally induced EIOP. Our results show that EIOP significantly impairs the presence of NGF and NGF-receptor proteins and TrkA gene expression in the ON of glaucomatous eye. These findings suggest that NGF and NGF-receptor might be important signals for the ON response in the EIOP.

Glaucoma (GL) is an ocular disorder characterized by progressive loss of visual field due to retinal ganglion cell (RGC) death and degeneration of the optic nerve (ON) fibers, usually in response to elevated intraocular pressure (EIOP) [23]. There is, however, evidences that altered environmental factors can contribute and/or exacerbate these degenerative events [11,25,27]. The neurotrophins, that include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3), are endogenously produced signaling molecules that play marked roles on specific cell population of peripheral sympathetic and sensory system and of brain neurons [3,9,19] and, as it has been demonstrated in a number of recent studies, also on cells of the visual system [7,18,26]. For example, NGF has been shown to exert a protective action on damaged ON of fish [6], amphibians [29], birds [10], and mammals [7,11,12], while abnormal expression of NGF, NGF-receptor proteins and of its mRNA has been detected in the ON of post-mortem subjects with multiple sclerosis [20] and in a rat model [2]. The functional role of NGF in the ON of glaucomatous eye is still largely unknown. Because GL induces marked damages in fibers and in cells of the ON cells, we investigated the response of NGF and NGF-receptors in the ON of experimentally induced GL in adult rats. Our results show that EIOP alters significantly the expression of NGF and the high-affinity NGF-receptor TrkA. These findings suggest that NGF and NGF-receptor are important signaling molecules for the ON and most likely for retinal cells.

Animal care and handling were in compliance in conformity with National and International laws (EEC Council Directive 86/609, OJ L 358, 1, December 12, 1987) and according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Unilateral elevation of IOP was induced in right eye of anesthetized adult male Sprague Dawley rats ($n = 20$) by injecting 50 μl of hypertonic saline into the superior episcleral vein, as described [21]. The left eye was used as non glaucomatous control rats. All experimental rats were housed in a constant low-light environment (40–90 lx) to minimize IOP circadian oscillations. Intraocular pressure was determined in awake animals using one drop of 0.5% proparacaine hydrochloride instilled in each eye. The calibrated TonoPen XL tonometer (Mentor, Norwell, MA) was used for daily monitoring of IOP. Animals with an IOP less than 30 mmHg were not classified as glaucomatous rats and were not used for this study. All rats were killed 5 weeks after induction of GL and retina and eye globe with ON removed and used for morphological, immunohistochemical and molecular analysis.

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The concentration of NGF was measured by a highly sensitive two-site enzyme immunoassay ELISA kit "NGF Emax™ Immunoassay System number G7631" by Promega (Madison, WI, USA), following the instructions provided by the manufacturer. The amount of NGF was determined from the regression line for the NGF standard (ranging from 7.8 to 500 pg/ml of purified mouse NGF), incubated under similar conditions in each assay and all assays were performed in duplicate.

The eye globe, including the ON were fixed in Bouin's fluid for 4 days, washed several times in 10% ammonium sulfate in 80% alcohol for 3 days to remove the fixative. The ON and retina were then individually sectioned with a cryostat at −20 °C and coded sections (10 μm thick) of the ON segments 1 mm from the back of the globe and retina were used for histological and/or immunohistochemical studies.

For histological analysis retina and ON sections were stained with hematoxilin–eosin and examined with a Zeiss Axiphot microscope with an objective 40× and a final magnification of 400×.

Other sections ($n = 10$) from each glaucomatous ON ($n = 5$) and control ON ($n = 5$) were immunostained with anti-TrkA, and anti-p75 as previously described [2,15]. Briefly, sections were first exposed to 3% of hydrogen peroxide (H_2O_2) and 10% of methanol W/V for 20 min followed by exposure of 0.1 M PBS containing 10% of horse serum for 1 h and then incubated overnight at 4 °C with antibodies against anti-TrkA, purchased from UPSTATE (Temecula, CA, USA). Sections were then exposed to biotinylated anti-rabbit IgG 1:300 (Vector Laboratories) with 2% of horse serum for 2 h at room temperature and then with avidin-biotinylated horseradish peroxidase complex (Vector Laboratories) in 0.1 M PBS with 0.1% Triton X-100 for another 2 h at room temperature followed by the treatment for 15′ with a 0.1 M solution of 3,3′-diaminobenzidine (DAB) (Sigma Chemical Company) in 0.1 M PBS 0.1% Triton X-100 containing, 0.025% cobalt chloride, and 0.02% nickel ammonium sulfate. All the section passed through all procedures simultaneously to minimize any difference from immunohistochemical staining itself. Staining specificity was assessed by omission of the primary antibodies.

Immunostained sections were observed under a Zeiss Axiophot microscope with an objective 100× under oil immersion and TrkA-positive cells counted in randomly selected, non-overlapping fields ($n = 12$) of each ON ($n = 5$) of control and glaucomatous eye ($n = 5$). For quantitative determination, we used an image processing analysis program Nikon-Lucia that automatically selects only cell bodies, but not small fragments or cells that do not have a complete soma and expressed as the number of immune-positive cells/300 mm^2 for ON area.

For ultra structural analysis, the ON head was fixed in 4% glutaraldehyde in 0.1 phosphate buffer, pH 7.2, for 24 h, washed with fresh buffer and then post-fixed in 1% OsO4 in the same buffer. The ON was then dehydrated with ascending ethanol and acetone and embedded in 812/Spurr's low-viscosity resin. Thin sections were then cut with an ultramicrotome and collected on 200 mesh grids. Sections were stained with uranyl acetate and lead citrate and viewed under an electron microscope.

For western Blotting Analysis of TrkA and p75 receptors, tissue samples were homogenized in lysis buffer (0.01 M Tris–HCl buffer, pH 7.4, containing 0.1 M NaCl, 1 mM EDTA, 1 mM EGTA, 2 mM PMSF, 50 μM leupeptin, 100 μg/ml pepstatin, and 100 μg/ml aprotinin) at 4 °C. After 8000 × g centrifugation for 20 min, the supernatants were used for Western blotting. Samples (30 μg of total protein) were dissolved with loading buffer (0.1 M Tris–HCl buffer, pH 6.8, containing 0.2 M DTT, 4% SDS, 20% glycerol, and 0.1% bromophenol blue), separated by 8% SDS-PAGE, and electrophoretically transferred to PVDF membrane for overnight. The membranes were incubated for 1 h at room temperature with blocking buffer (5% non-fat dry milk, 10 mM Tris, pH 7.5, 100 mM NaCl, and 0.1% Tween-20). Membranes were washed three times for 10 min each at room temperature in TTBS (10 mM Tris, pH 7.5, 100 mM NaCl, and 0.1% Tween-20) followed by incubation at 4 °C with primary antibodies over night. Membranes were washed three times for 10 min each at room temperature in TTBS and incubated for 1 h with either horseradish peroxidase-conjugated anti-rabbit IgG or horseradish peroxidase–conjugated anti-mouse IgG as the secondary antibody. The blots were developed with ECL chemiluminescent HRP substrate (Millipore Corporation, Billerica, USA) as the chromophore. A personal computer and the public domain (http://rsb.info.nih.gov/ij/) Image J software were used to evaluate band density, which was expressed as arbitrary units of grey level. The ImageJ software determines the optical density of the bands using a grey scale thresholding operation. The optical density of β-actin bands was used as a normalizing factor. For each gel/blot, the normalized values were then expressed as percentage of relative normalized controls and used for statistical evaluation.

For RT-PCR analysis of TrkA and p75, total RNA from retina and ON was extracted using Versagene™ RNA tissue kits (Gentra Systems Inc.). RNA was quantified by spectrophotometer at 260 nm. RNA was converted into cDNA in a 25 μl reverse transcription reaction containing 2-γ of total RNA. Reactions were incubated at 42 °C for 60 min, heated at 95 °C for 5 min, then cooled at 4 °C for a minimum of 5 min and a maximum of 30 min.

The polymerase chain reaction (PCR) was analyzed using the 7900HT Fast Real-Time PCR System (PE Applied Biosystems) and FAM-labeled probe specific for the NTrk1 (NM 021589.1), and Ngfr (NM 012610.1) (PE Applied Biosystems). Designed primers and a FAM-labeled probe for rat ACTB (actin, beta) (4352931E) were included in the reactions as endogenous control. The cDNA was amplified under the following conditions: 1 cycle at 50 °C for 2 min and at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and at 60 °C for 1 min. The amount of mRNA of each gene was calculated using the standard curve method and adjusted for the expression of ACTB. The data have been presented as percentage of control groups.

Statistical evaluations were performed using the StatView package for Windows and data were expressed as mean and S.E.M. TrkA and p75 optical density data from Western blot analyses were evaluated using the analysis of variance (one-way ANOVA) followed by Tukey–Kramer post hoc test. A p-value less than 0.05 was considered significant.

As previously reported [2,15], intraocular administration of hypertonic saline into the episcleral vein of adult rats increases the IOP. A week after this treatment the IOP was 42.7 ± 3.7 mmHg compared to 26.9 ± 2.6 mmHg of control eye. The IOP remains elevated for 5 weeks, the last point-time checked.

Animals were killed 5 weeks following the surgical procedure. Histological analysis, shown in Fig. 1, indicated that EIOP causes reduction of cells in the retinal ganglion layer (B) and in the ON (D), compared to their respective controls, Fig. 1A and C. Moreover, detailed ultrastructural studies revealed that EIOP causes axon swelling and myelin debris, as indicated with arrows in Fig. 1E and F. Immunohistochemical studies showed the presence of TrkA stained cells in the ON, Fig. 1G and H. A marked reduction in the presence of TrkA stained cells was found in the ON in EIOP, Fig. 1H as compared to the control rats, Fig. 1G.

Quantitative analysis of immunostained TrkA-positive cells indicates that this decrease is statistically significant ($p < 0.01$), Fig. 1I. No differences in p75 immunostained cells between control and glaucomatous ON nerve were found (data not shown). The result of the immunoenzymatic assay, reported in Fig. 1L, revealed that the EIOP also causes a significant ($p < 0.01$) reduction of NGF protein in the ON.

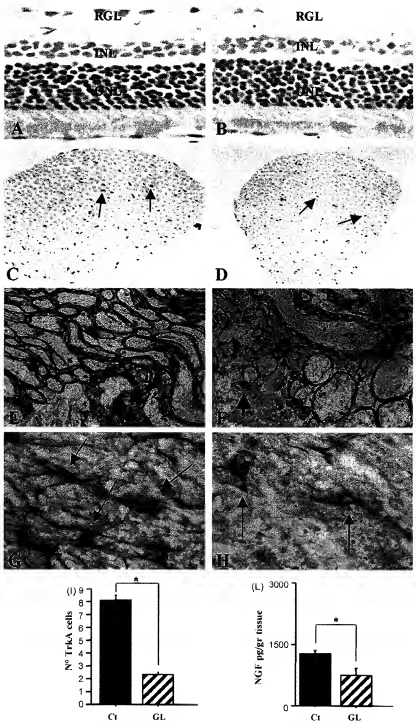

Fig. 1. Sections of control (A) and glaucomatous (B) retina showing cell reduction in the retinal ganglion layer (RGL) in the eye of rat with EIOP. INL, inner nuclear layer; ONL, outer nuclear layer. Magnification 210×. Representative histological preparations of control (C) and of glaucomatous ON (D). Note that the presence of cells (arrows) in the ON of glaucomatous rats less numerous compared to control ON. Magnification 60×. Representative electron microscopic fields of control (E) and glaucomatous ON (F) showing axon swelling (arrows), and myelin debris (thick arrow) in the glaucomatous ON. Magnification 35,000×. Representative TrkA-immunostained sections of control (G) and glaucomatous ON (H). Note the marked reduction of immunostained TrkA cells (arrows) in glaucomatous ON. Magnification 300×. (I) Quantitative evaluation of TrkA-immunostained cells in control (Ct) and glaucomatous (GL) ON. Note the statistical significant ($p < 0.01$) reduction of TrkA-positive cells in glaucomatous ON. (L) Level of NGF protein in the ON of control (Ct) and glaucomatous (GL) indicating reduced presence ($p < 0.01$) of NGF in the ON glaucomatous eye.

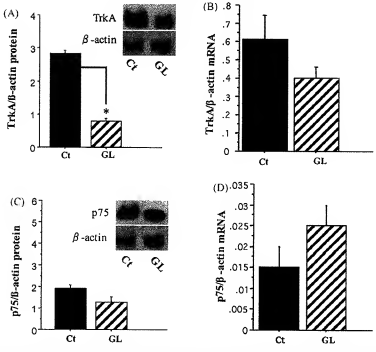

Fig. 2. TrkA protein (A) and TrkA-mRNA (B) expression in the ON of control (Ct) and glaucomatous (GL) ON. Note the marked reduction, particularly of TrkA protein in glaucomatous ON. p75 (C) protein and p75-mRNA (D) in the ON of control (Ct) and glaucomatous (GL) ON. No significant differences were found in the expression of p75 protein and gene expression, between control and glaucomatous ON.

As reported in Fig. 2, western blot and RT-PCR studies indicated that EIOP reduces the presence of TrkA protein (Fig. 2A,) and TrkA gene expression (Fig. 2B) in the ON. EIOP has no effect on p75 protein (Fig. 2C), and gene expression (Fig. 2D).

Results from the present study indicate that EIOP induced by injection of hypertonic saline into the episcleral vein reduced the presence of NGF in the ON. EIOP reduces the number of TrkA-positive cells in the ON, compared to control ON, and causes no changes in the p75 expression. Moreover biochemical and molecular analyses indicated that EIOP reduces the presence of TrkA protein and TrkA gene expression, but not p75 protein and gene expression. The effect of EIOP is associated with widespread axonal swelling in the ON, as reported by others [21]. The functional significance of the different expression in NGF-receptors in the glaucomatous eye is not clear. NGF is known to promote cell survival in the presence of TrkA, while p75 has been shown to promote apoptosis under conditions where TrkA is reduced or absent [8]. Thus, the low-affinity NGF-receptor, p75, can be involved in cell death and cell survival, depending on a number of biochemical and molecular variables. It is therefore possible that the different response of these receptors in glaucomatous ON is most likely related to these variable mechanisms. Another possibility is that within the ON, TrkA and p75 are independently regulated. For example, in the brain the expression of p75 is not associated with apoptotic mechanism [22].

The major cell components of the ON are the oligodendrocytes that play an important role in supporting the functional activity of ON axons [24]. These cells produce and release NGF [4,5,20,29] and seem to be able to regulate the synthesis and activity of NGF through autocrine mechanisms [28]. However, whether the altered NGF and TrkA expression is the result of structural changes of the ON induced by EIOP or vice versa needs to be further investigated.

The hypothesis of a functional role of NGF in the ON is supported by evidences showing that NGF and its receptors are expressed in fish, amphibian, and mammalian ON [6,10,29], including the ON of subjects affected by multiple sclerosis [20] and that their presence is important during axonal regeneration, and damaged ON. Other findings showing that NGF promotes retinal cellular growth and differentiation [6,29,30], reduces retinal cell damage due to ocular ischemia [26], protects damaged retinal cells death induced by ocular hypertension [14] and that intraocular administration of NGF reduces RGC degeneration following rat ON lesions [7] are consistent with this hypothesis.

Based on the above observations we speculate that down-regulation of the high-affinity NGF-receptor in combination with reduced presence of NGF may contribute to the progression of ON axon degeneration in glaucomatous eye. These findings may have important clinical bearing as NGF treatment previously has been applied with success in a variety of eye disorders [1,13,16,17]. Further studies are planned to investigate the effect of NGF treatment in EIOP induced ON neuropathy.

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Visual field assessment in glaucoma: Comparative evaluation of manual Kinetic Goldmann Perimetry and Automated Static Perimetry

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Abstract

Purpose: To compare the detection and assessment of progression of visual field defects in primary open-angle glaucoma with manual suprathreshold perimetry on Goldmann perimeter and automated static threshold perimetry on Humphery visual field (HVF) analyzer. **Methods:** 105 eyes of 54 patients of primary open-angle glaucoma were followed up with 3-monthly perimetry on Goldmann perimeter and HVF analyzer, for a period of 9 months. **Results:** HVF analyzer picked up visual field defects in 48 (46%) eyes whereas Goldmann perimeter picked up visual field defects in 26 (25%) eyes. HVF analyzer demonstrated progression in 14 eyes whereas Goldmann perimeter detected progression in 7 eyes during follow up of 9 months. **Conclusions:** HVF analyzer is superior to Goldmann perimeter to document and to demonstrate progression of visual field defects in primary open-angle glaucoma.

Keywords: Adolescent, Adult, Aged, Aged, 80 and over, Automatic Data Processing, Comparative Study, Female, Glaucoma, Open-Angle, diagnosis, physiopathology,

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The most useful parameters for evaluation of glaucoma status in primary open-angle glaucoma are intraocular pressure (IOP), optic disc cupping and visual field assessment. Perimetry is an objective

method of assessing the visual damage; therefore, visual field assessment through perimetry is important in the diagnosis and management of primary open-angle glaucoma. Prior to the advent of automated perimetry, the Goldmann perimeter was used to detect visual field defects and their progression. It is rapid and covers a wide area. The installation and maintenance cost is not high. However, the perimetrist's involvement in measuring and assessing the visual fields on the Goldmann perimeter may introduce an element of subjectivity; this remains the chief drawback of manual perimetry. The superiority of the Goldmann perimeter is adequately supported in the literature.[1-5] Automated perimeters are objective, accurate and supported by useful software packages to assist in the assessment of the visual fields. At the same time, the cost of objectivity is high in financial terms; the Humphrey visual field analyzer costs about Rs. 200,000 more than the Goldmann perimeter at the time of the study. Automated perimeters have now mustered adequate support in ophthalmic literature.[6-10] Several studies have highlighted the usefulness of both methods of perimetry. [11,12] However, in India, resources being limited, cost is an important consideration. Hence, the two methods of perimetry were compared with regard to detection and assessment of progression of visual field defects.

Materials and Methods

Fifty-four patients were randomly selected from the glaucoma clinic or the outpatient department of the Rajendra Prasad Centre for Ophthalmic Sciences, New Delhi, India. They were categorized as primary open-angle glaucoma if the IOP was > 21 mm Hg with glaucomatous disc changes and /or visual field defects of glaucoma and a widely open angle on gonioscopy. The diagnosis of ocular hypertension was established if the IOP was > 21 mm Hg on two consecutive occasions without optic disc and visual field changes of glaucoma. A value of 21 mm Hg as a cutoff has been considered statistically valid.[13] Cases with other types of glaucoma, visual field loss due to causes other than glaucoma and eyes with a best corrected visual acuity less than 6/12 were excluded from the study.

In all patients a detailed clinical history of frequent change of presbyopic correction, impaired dark adaptation and visual field defects was recorded. A history of past and present medical therapy and surgical/laser treatment of glaucoma was noted. A complete ocular anterior segment slitlamp examination was conducted in oblique illumination under magnification. Fundus examination was performed with a direct ophthalmoscope to document the degree of optic disc cupping and to rule out any other lesion of the optic disc or the retina. Stereobiomicroscopic examination of the disc was done through the contact lens of the gonioscope at the time of gonioscopy, but a +90 D examination was not done. Refraction with/without cycloplegia was conducted to obtain the best corrected visual acuity for near and distance. IOP was measured using the Goldmann applanation tonometer. Diurnal variation of IOP on topical anti-glaucoma therapy was recorded. Gonioscopy was done to confirm the open status of the angle and to rule out other types of glaucoma. During follow up, random applanation tonometry was performed. If the IOP was recorded above 21 mm Hg a repeat diurnal was recorded and the anti-glaucoma therapy was adjusted to lower the IOP below 21 mm Hg.

Visual field assessment was done first on the Goldmann perimeter (model S-940, Haag Streit, Berne, Switzerland) followed by Humphrey visual field analyzer (model 735, Humphrey Instruments, Carl Zeiss, Germany). The visual field assessment on both perimeters was carried out on the same day. Patients on topical pilocarpine were advised to stop instillation of the drug 48 hours prior to examination and were temporarily put on oral acetazolamide, 250 mg four times a day to ensure a pupil size of more than 2.5 mm. A second visual field examination was done on

HVF analyzer after two days. In case of improvement, original defect was considered as learning effect and another visual field examination was done. On obtaining two successive consistent visual fields, the learning effect was deemed to be eliminated. Also, in case of poor patient cooperation due to fatigue or any other reason leading to unreliable fields, Humphrey visual fields were obtained on a subsequent date. Patients with consistently unreliable visual fields were not included in the study.

Visual field assessment on Goldmann perimeter was done by kinetic method with suprathreshold checks. Goldmann perimetry was performed on all cases by a single physician (VG). Three isopters l-4e, l-3e and l-2e were initially plotted for all patients. Near correction was used in the presbyopic age group and the requisite refractive correction in others before charting the visual field. Fixation was monitored throughout using the telescope.

Visual field assessment on the Humphrey visual field analyzer was done using full threshold program 30-2. A size III white stimulus was used for the assessment. The entire test was supervised and the patient was actively encouraged to maintain fixation throughout the test. A printout of the visual fields was obtained at the end of the examination. The visual field examination was done at intervals of 0, 3, 6 and 9 months.

Goldmann fields were analyzed for presence of any visual field defect (VFD) and its progression by one consultant physician (HCA/RS). On Goldmann perimeter, the criteria for detection and progression of a visual field defect were the same as used by Chauhan et al[13] [one or more scotomas with a minimum width of 5°, a horizontal nasal step with one isopter (or the sum of steps with multiple isopters) of at least 10°, presence of sector-shaped defect]. Each field defect was counted as an individual field defect. For example, a nasal step and a paracentral scotoma on a field were counted as two visual field defects on that field. The progression on Goldmann perimeter was defined using the criteria outlined by the same author [appearance of any of the above on a previously normal field; kinetic enlargement of a scotoma by at least 5°, in any direction or increase in horizontal nasal step with 1 isopter (or the sum of steps with multiple isopters) of at least 10°].

On HVF analyzer, the criteria used for detection and progression of a visual field defect were the same as that described by Anderson[14] [pattern deviation plot showing a cluster of 3 or more non-edge points at an expected location that have sensitivities occurring in fewer than 5% of the normal population ($p<5\%$), one of the points having a sensitivity that occurs in fewer than 1% of the population ($p<1\%$) with CPSD having $p<5\%$; and GHT outside normal limits]. A field was considered to have a visual field defect (VFD) if it satisfied the criteria used for the purpose of the study. If more than one location on the field satisfied the criteria, the field defects were counted accordingly. However, no statistical program was used to determine progression. Progression[15] included the appearance of a new defect in accordance with the definition of defect used for the purpose of the study and was confirmed on at least one subsequent field; increase in depth of the defect by at least 0.5 log units (5 dB) in pattern deviation on at least 3 contiguous locations confirmed on at least one subsequent field or increase in depth of the defect by at least 1 log units (10 dB) with an increase in depth of the defect by at least 0.5 log units (5 dB) in pattern deviation on a contiguous location. The trend towards progression was confirmed on at least one subsequent field.

Results

A total of 54 patients (28 males; 26 females) were included in the study. All patients had

involvement of both eyes. Both eyes of 51 patients and one eye in 3 patients (the other eye had poor vision) were included in the study, making a total of 105 eyes. Of these, 52 were right and 53 were left eyes.

The mean age of the sample population was 50.59 ± 15.81 years. The youngest patient was 14 years old and the oldest patient 82 years. Thirty patients (57 eyes) had a diagnosis of primary open-angle glaucoma; 17 patients (34 eyes) had ocular hypertension and 7 patients (14 eyes) had juvenile open-angle glaucoma. At the time of inclusion into the study, the mean IOP was 18.42 ± 3.16 mm Hg on anti-glaucoma medications. In eyes with IOP>21 mm Hg, anti-glaucoma medication was increased to bring the IOP below 21 mm Hg. During follow up, all patients had IOP<21 mm Hg with topical medications. The number of eyes controlled on one, two and three topical medications were 66, 35 and 4 respectively. The mean cup-disc ratio for the population was 0.53 ± 0.20.

At initial presentation [Figure - 1], Goldmann perimetry detected visual field defects in 23 (22%) eyes. HVF analyzer showed field defects in all these eyes; an additional 21 (20%) showed field defects on HVF analyzer alone, taking the tally of eyes with visual field defects on HVF analyzer to 44 (42%). By 3 months [Figure - 2], the same 23 (22%) eyes showed field defects both on Goldmann perimetry and HVF analyzer whereas 22 (21%) eyes showed field defects on HVF analyzer alone, taking the tally of eyes with visual field defects on HVF analyzer to 45 (43%). By 6 months [Figure - 3] 24 (23%) eyes showed field defects on Goldmann perimetry. HVF analyzer showed field defects in all these eyes; an additional 23 (22%) showed field defects on HVF analyzer alone, taking the tally of eyes with visual field defects on HVF analyzer to 47 (45%). By 9 months [Figure - 4], 26 (25%) eyes showed field defects on Goldmann perimetry. On HVF analyzer, besides these eyes, 22 (21%) additional eyes showed field defects. Hence at the end of the follow-up period, 48 (46%) eyes showed field defects on HVF analyzer.

For eyes with 0.3 to 0.4 cupping, Goldmann perimeter did not pick up visual field defects in any of the patients whereas HVF analyzer picked up visual field defects in 6 of 44 (14%) eyes at presentation. By 9 months, in the same subgroup, HVF analyzer picked up visual field defects in 8 (18%) eyes, whereas Goldmann perimeter still failed to show the presence of any field defects.

For eyes with 0.5-0.6 cupping both Goldmann perimetry and HVF analyzer showed a field defect in 5 of 29 (17%) eyes whereas HVF analyzer picked up visual field defects in another 8 (28%) eyes, i.e., the total number of eyes with visual field defects on HVF analyzer was 13 (45%). In the same subgroup, by the time of the 9-month follow-up visit, both Goldmann perimetry and HVF analyzer showed a field defect in 8 (28%) eyes, whereas HVF analyzer picked up visual field defects in 7 (24%) extra eyes; taking the tally of eyes with 0.5-0.6 cupping with visual field defects on HVF analyzer to 15 (52%).

With 0.7-0.8 cupping both Goldmann perimetry and HVF analyzer picked up glaucomatous visual field defects in 12 of 26 (46%) eyes, whereas in the same patients, the HVF analyzer picked up visual field defects in 7 (27%) additional eyes [a total of 19(73%)] at presentation. In this subgroup the figures remained the same by the 9-month follow-up visit.

For cupping of more than 0.8 both Goldmann perimeter and HVF analyzer picked up visual field defects in 6 of 6 (100%) eyes at presentation as well as at the 9-month follow-up period [Figures:1-4]. HVF analyzer picked up all eyes with visual field defects that were picked up by Goldmann perimeter. The other eyes with visual field defects picked up by HVF analyzer were in addition to those picked up by Goldmann perimeter.

At presentation [Figure - 1], Goldmann perimetry picked up 25 glaucomatous visual field defects in

23 eyes (6 nasal steps, 6 sector defects and 13 arcuate scotomas), which increased to 30 glaucomatous visual field defects in 26 eyes (7 nasal steps, 9 sector defects and 14 arcuate scotomas) by the end of the 9-month follow up [Figure - 4]. HVF analyzer at presentation picked up 56 visual field defects in 44 eyes (19 in paracentral area, 15 in nasal step area, 5 sector defects and 17 in arcuate area) which increased to 69 visual field defects in 48 eyes (28 in paracentral area, 17 in nasal step area, 4 sector defects and 20 in arcuate area) by 9-month follow up. The paracentral defect which was demonstrated on HVF analyzer could not be detected on Goldmann perimetry in the same eye at the same time [Table - 1].

At presentation [Figure - 1], in eyes with 0. 3-0. 4 cupping Goldmann perimetry detected no visual field defects (VFDs) whereas HVF analyzer picked up 7 VFDs in 6 eyes (3 in paracentral area and 4 in nasal step area). By the end of the 9-month follow-up [Figure - 4] Goldmann perimetry continued to show the absence of any field defect whereas the number of VFDs on HVF analyzer increased to 10 (6 in paracentral area and 4 in nasal step area) in 8 eyes. In eyes with 0.5-0.6 cupping, at presentation, Goldmann perimetry picked-up 5 VFDs in 5 eyes (2 nasal steps, 1 sector defect and 2 arcuate scotomas) whereas HVF analyzer picked up 18 VFDs in 13 eyes (9 in paracentral area, 3 in nasal step area, 3 sector defects and 3 in arcuate area). By 9 months in the same subgroup Goldmann perimetry picked up 9 VFDs in 8 eyes (3 nasal steps, 3 sector defects and 3 arcuate scotomas) whereas HVF analyzer picked up 23 VFDs in 15 eyes (14 in paracentral area, 3 in nasal step area, 2 sector defects and 4 in arcuate area). In eyes with 0.7-0.8 cupping Goldmann perimetry picked up 12 VFDs in 12 eyes (1 nasal steps, 3 sector defect and 8 arcuate scotomas) whereas HVF analyzer picked up 23 VFDs in 19 eyes (7 in paracentral area, 5 in nasal step area, 2 sector defects and 9 in arcuate area) at presentation. By 9 months in the same subgroup Goldmann perimetry picked up 12 VFDs in 12 eyes (1 nasal steps, 3 sector defect and 8 arcuate scotomas) whereas HVF analyzer picked up 27 VFDs in 19 eyes (7 in paracentral area, 7 in nasal step area, 2 sector defects and 11 in arcuate area). At presentation, in eyes with more than 0.8 cupping Goldmann perimetry picked up 8 VFDs in 6 eyes (3 nasal steps, 2 sector defects and 3 arcuate scotomas) whereas HVF analyzer also picked up 8 VFDs in 6 eyes (3 in nasal step area and 5 in arcuate area). By 9 months, in the same subgroup, Goldmann perimetry picked up 9 VFDs in 6 eyes (3 nasal steps, 3 sector defect and 3 arcuate scotomas) whereas HVF analyzer also picked up 9 VFDs in 6 eyes (1 in paracentral area, 3 in nasal step area and 5 in arcuate area) [Table - 1].

We found that at presentation, Goldmann perimetry picked up 25 visual field defects in 23 eyes (from 105 eyes), whereas HVF analyzer picked up 56 visual field defects in 44 eyes. At the 3-months follow up Goldmann perimetry picked up the same 25 visual field defects in 23 eyes, whereas HVF analyzer, at the same time picked up 56 visual field defects in 45 eyes. By the 3-month follow-up visit one eye had shown progression of visual field defects on both Goldmann perimeter and HVF analyzer whereas two additional eyes had shown progression of visual field defects on HVF analyzer alone. Similarly at the 6-month follow-up visit 3 eyes had shown progression of visual field defects on both Goldmann perimeter and HVF analyzer whereas 5 additional eyes had shown progression of visual field defects on HVF analyzer alone. By the 9-month follow-up visit 7 eyes had shown progression of visual field defects on both Goldmann perimeter and HVF analyzer whereas 7 additional eyes had shown progression of visual field defects on HVF analyzer alone [Table - 2].

Seven eyes that had shown progression of visual field defects on Goldmann perimetry also showed progression of visual field defects on HVF analyzer. The two sets of visual fields were correlated temporally as shown in [Table - 3]. Up to the 3-month follow up of these 7 eyes, one had shown progression of visual field defects on Goldmann perimetry whereas three had shown progression of visual field defects on HVF analyzer. By the 6-month follow up of these 7 eyes, three had shown progression of visual field defects on Goldmann perimetry whereas all 7 eyes had shown

progression on HVF analyzer. By the end of the 9-month follow-up, the remaining 4 eyes showed progression of visual field defects on Goldmann perimetry as well.

Discussion

Visual field assessment is mandatory for the diagnosis and management of primary open-angle glaucoma. The Goldmann perimeter is widely available, economical and easy to maintain. But it requires frequent calibrations and highly skilled technicians to do the visual field examination; also, it does not measure the depth of a scotoma. It gives a rapid, comprehensive coverage of the entire field and produces recognizable isopter patterns.[1] At the same time, it fails to detect the early diffuse loss of retinal sensitivity. It works well for the definition of the topography of the visual field defects and subsequent progression, but is less efficient in the detection of small field defects. It is more patient friendly, as patients find it less tiring and easier to maintain fixation.[5] However, there is the possibility of observer bias and it requires the technician's deep involvement in the assessment of the visual field. The automated perimeter eliminates observer bias. The test is easier and can be performed by less skilled technical staff. However, the equipment is more expensive to purchase and maintain. Automated perimetry is for defining the depth of a scotoma and progression in depth of a visual field defect. It is superior for detection of generalized depression of retinal sensitivity, which forms the earliest visual field defect and is often missed by the Goldmann perimeter.[12] This is partially offset by the fact that the visual angle between static locations in the commonly used programs is more than the upper limits of translocation error on kinetic perimetry, making the topography of field defects a statistical interpolation rather than actual measurement. The statistical analysis by the software in an automated perimeter is more quantitative and accurate, but at the same time the patient finds the test procedure more tiring and the maintenance of fixation during visual field charting more difficult.[5] In the literature there is no consensus regarding the superiority of one perimeter over the other and each of these has been found to have distinct advantages and disadvantages influenced by the stage of the disease and other variables of the examination procedure. [1, 4, 8, 12]

In our study, the automated perimeter picked up visual field defects in a larger number of eyes than the Goldmann perimeter. The difference was greatest for eyes with early cupping, which narrowed down progressively with increasing cup-disc ratio. Visual field defects were more extensive on automated perimetry compared to Goldmann perimetry. The paracentral area visual field defects picked up by the HVF analyzer failed to show up on the Goldmann perimeter. The other area of major discrepancy between the two perimeters was the nasal step. The number of visual field defects in the nasal step area picked up by HVF analyzer was about 2.5 times as many as on Goldmann perimeter. The discrepancy for sector defects and arcuate area defects was less between the fields obtained by the two methods of perimetry.

HVF analyzer picked up progression in twice as many eyes as compared to the Goldmann perimeter during a follow up of 9 months. The superiority of HVF analyzer has clinical significance, because in a disease with largely irrecoverable visual field loss like glaucoma, it is important to pick up progression of visual field defects early so that the treatment may be modified or altered to prevent further visual field loss. The advantage of the HVF analyzer also lies in its ability to make use of quantified parameters like mean deviation and corrected pattern standard deviation to detect subtle worsening of visual field defect, with statistical level of confidence. This is beyond the detection capacity of the Goldmann perimeter. We conclude that automated HVF analyzer is superior to the Goldmann perimeter in detecting early glaucomatous visual field defects.[15]

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Figures

[Figure - 1], [Figure - 2], [Figure - 3], [Figure - 4]

Tables

[Table - 1], [Table - 2], [Table - 3]

This article has been cited by

1 **Narrow vision after view-broadening travel**

De Mendonça Melo, M., Martinez Ciriano, J.P., Van Genderen, P.J.J.
Journal of Travel Medicine. 2008; 15(4): 278-280
[Pubmed]

2 **Open-angle glaucoma: Variations in the intraocular pressure after visual field examination**
Recupero, S.M., Contestabile, M.T., Taverniti, L., Villani, G.M., Recupero, V.
Journal of Glaucoma. 2003; 12(2): 114-118
[Pubmed]

Clinical assessment of optic nerve disorders

Riordan-Eva P. Eye (2004) 18, 1161–1168

The Optic Neuritis Treatment Trial highlighted the seemingly different results obtained with computerised static perimetry, although this may simply reflect differences in interpretation.[22] Diffuse loss on Humphrey 30-2 is probably equivalent to a central scotoma on Goldmann perimetry. Similarly, on computerised perimetry eccentric fixation may influence the pattern of visual field defect. It is necessary for clinicians to gain experience for each visual field testing technique, as well as bearing in mind that visual field defects are not pathognomonic.
For monitoring optic neuropathy, computerised perimetry is the best available technique, providing sensitivity, reproducibility, and concentrating on the central visual field. It also provides correlation to retinal ganglion cell loss.[23,24]

Understanding Visual Fields, Part I; Goldmann Perimetry

Dersu I et al. Journal of Ophthalmic Medical Technology (2006) 2; 2.

Advantages and Disadvantages

During Goldmann perimetry, dimmer stimuli are used for testing the very center of vision with the intensity increasing as more peripheral portions of the field are tested.
Some patients might prefer it because there is human interaction. By the same token, it is very much examiner dependent[1]. It may not be reproducible by another examiner, and it does not have the advantages of a computerized system for storage and comparison to normative data. Additionally, kinetic perimetry may not be as sensitive as static perimetry in detecting early glaucoma defects[3]. However, Goldmann visual fields might reveal scotomas that were missed between the testing points in static perimetry[4]. The shape of the defects may also be more impressive in Goldmann perimetry1. With severe vision loss (vision worse than 20/200), test-retest variability might be better in comparison to automated static testing. In addition, it shows functional (non-organic) defects on visual field testing better than automated testing.

Comparison between Semiautomated Kinetic Perimetry and Conventional Goldmann Manual Kinetic Perimetry in Advanced Visual Field Loss

Nowomiejska K et al. Ophthalmology Vol 112, Issue 8, August 2005, Pages 1343-1354

However, there are many disadvantages of the Goldmann instrument used for these purposes. Direction and speed of stimulus movement are guided by the examiners' hand and, therefore, are difficult to standardize. Thus, the results depend on the examiner's skills and may be confounded by examiner bias.[9] Examiner dependence can be associated with inaccuracy, which results in a limited capacity to detect defects and poor reproducibility of results.[10] Kinetic VF results, as being subjectively obtained, are notoriously difficult to quantify, and this is made even more difficult by the lack of standardization of equipment and method.[11] Because of the pantograph mechanism in the Goldmann instrument, the spatial resolution decreases with increasing eccentricity, which can give rise to a poor cartographic accuracy.[12] There are also other shortcomings of the Goldmann perimeter, such as lack of autocalibration, lack of permanent documentation of the test procedure

used to determine individual VF borders, and the inability to examine the area of 2° around the fixation point with the standard setting due to the telescope used for fixation control. In principle, this problem can be solved by an alternate fixation target, but it is still a difficult process.
To overcome disadvantages of Goldmann perimetry, a new software-based technique (Invest Ophthalmol Vis Sci 41:295, 2000) called semiautomated kinetic perimetry (SKP) was designed…

Visual field assessment in glaucoma: Comparative evaluation of manual Kinetic Goldmann Perimetry and Automated Static Perimetry

HC Agarwal et al. Indian Journal of Ophthalmology (2000). 48:4;301-6.

Visual field assessment is mandatory for the diagnosis and management of primary open-angle glaucoma. The Goldmann perimeter is widely available, economical and easy to maintain. But it requires frequent calibrations and highly skilled technicians to do the visual field examination; also, it does not measure the depth of a scotoma. It gives a rapid, comprehensive coverage of the entire field and produces recognizable isopter patterns.[1] At the same time, it fails to detect the early diffuse loss of retinal sensitivity. It works well for the definition of the topography of the visual field defects and subsequent progression, but is less efficient in the detection of small field defects.

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